A Thesis On

BIO-TRANSFORMATION OF NATURAL OILINTO INDUSTRIALLY USEFUL PRODUCT (LAURIC ACID)

Submitted By

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(610CH307)

Under the Supervision of

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Certificate

*Certified that this Project thesis entitled "**Bio-transformation of natural oil into industrially useful product (Lauric acid)**" by **V.Balaji Patro(610 CH 307)** during the year 2011 - 2013 in partial fulfillment of the requirements for the award of the Degree of Master of Technology (Research) in Chemical Engineering at National Institute of Technology, Rourkela has been carried out under my supervision and this work has not been submitted elsewhere for a degree.*

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20th January, 2013 **V.Balaji Patro**

Rourkela

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Nomenclatures

γ	Overall Degree Of Standardized Deviation
Δ_{max}	Global Maximum
Δ_{min}	Global Minimum
ξ	Distinguished Coefficient
ξ_i	Grey Relation Coefficient
% D-	Percentage Degradation
τ-	Space time
ABI-	Applied Biosystem
AM-	Antimicrobial
ANOVA-	Analysis of variance
ATP-	Adenosine triphosphate
ATW-	Atmospheric thin window
BDT-	BigDye Terminator
BLAST-	Basic local alignment search tool
BSA-	Bovine serum albumin
CMO-	Customer manufacturing organization
CSTR-	Continuous stirred tank reactor
CTAB-	Cetyl trimethylammonium bromide
DHA-	Docosahexaenoic acid
DMSO-	Dimethylsulfoxide

DNA-	Deoxyribonucleic acid
dNTP-	Deoxynuleoside triphosphate
DO-	Dissolve oxygen
DSP-	Downstream processing
E_{24}-	Emulsification Index at 24 Hours' Time
EDTA-	Ethylene diamine tetra acetic
EPA-	Eicosapentaenoic acid
FTIR-	Fourier transform infrared spectroscopy
GC-MS-	Gas Chromatography Mass spectrometry
GRC-	Grey Relational coeffecient
GRG-	Grey Relational Grade
HDL-	High density lipoprotein
IPTG-	Isopropyl-b-D-thiogalactopyranoside
ISPR-	In-situ product removal
IU-	International Unit
LBT-	Luria-Bertani-Tributyrin
LCFA-	Long chain fatty acid
LCT or LCTs-	Long chain triglycerides
LDL-	Low Density lipoprotein
MCF-	Medium chain fatty acid
MCFA-	Medium Chain Fatty acid
MCFA-	Medium chain fatty acid

MCS-	Multiple cloning site
MCSFA-	Medium chain saturated fatty acid.
MCT or MCTs -	Medium chain triglyceride
MSM-	Minimal Salt media
NCBI-	National center for Biotechnology Information
NLF-	Non lactose fermentation
NPDES -	National Pollutant Discharge Elimination System
OD-	optical density
OECD-	Organisation for Economic Co-operation and Development
PCR-	Polymerase chain reaction
PD-	Primer dimer
PFR-	Plug flow reactor
PUFA-	Polyunsaturated fatty acid
RDP-	Ribosomal Database project
RE-	Restriction enzyme
RPM-	Revolutions per minute
SCFA-	Short chain fatty acid
SDS-	Sodium dodecyl sulfate
SEM-	Scanning electron microscope
t-	Time
TAE-	Tris acetate EDTA
Taq-	Thermus aquaticus

U-	Unit
UV/Vis-	Ultra violet/Visible
VLCFA-	Very long chain fatty acid
W	Wave length (nm)
X-gal-	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

Abstract:

The production of industrially useful product from coconut oil by the biotransformation process with the help of microbes is a new alternative. Biotransformation studies were conducted using bacterial strain isolated from Rushikulya river beach, Ganjam, Odisha. The isolated microbial strain was characterized and confirmed as *Pseudomonas aeruginosa* through biochemical and 16s-rDNA analysis. The process parameters were optimized for both aerobic and anaerobic condition. The growth kinetic study exhibited a long lag phase in anaerobic condition as compared to aerobic condition. Lipase estimation and optimization were carried out by using standard 4-nitrophenol method with suitable substrate. Transformation studies with coconut oil as sole substrate produced fatty acids (lauric acid & its derivatives and others like glycerol, mystric acid in traces) under anaerobic condition. Fatty acid production was confirmed using GC-MS analysis. The product and substrate kinetics for lauric acid production was studied. Lipase gene was isolated from *pseudomonas sp* and confirmed. The antimicrobial activity of different concentration of lauric acid was studied using gram (+ve) and gram-(ve) bacterial sp.

Keywords: Biotransformation, aerobic, anaerobic, lauric acid

Dedicated to

My Parents…

CHAPTER-1

INTRODUCTION

1.1 Prelude:

In the coming post petrochemical period, production processes will be required to save energy and to reduce environmental damage. In this sense, biological reactions are now widely recognized as practical alternatives to conventional chemical reactions [KM Koeller et al. 2001, A. Schmid et al. 2001]. On the other hand, novel catalytic procedures are necessary to produce the emerging classes of organic compounds that are becoming the targets of molecular and biomedical research. Therefore, screening for novel biocatalysts that are capable of catalyzing new reactions is constantly needed. A bioprocess is sometimes designed from an organic chemistry standpoint, regardless of whether or not a suitable biocatalyst has already been found. This forces screening from a certain level to ascertain the existence of a desirable biocatalyst. One of the most efficient and successful means of finding new biocatalysts is to screen large numbers of microorganisms, because of their characteristic diversity and versatility [H Yamada et al. 1988, S Shimizu et al. 1997, J Ogawa 1999 & 2002]. Usually, screening is simply focused on a one-step target reaction, In which the cells of microorganisms are incubated with target substrates under various reaction conditions and their transformation is monitored.

Biotransformation is the process whereby a substance is changed from one chemical to another (transformed) by a chemical reaction mediated by microorganisms or enzymes [National library of Medicine (NLM)]. Although biotransformation has become an established method in organic chemical synthesis[A. Schmid et al. 2001] biocatalysis could, from our perspective, have a much bigger impact in this area. Fermentation processes, combined with traditional recovery methods, are emerging as a successful path to bio based products from natural oil. Because of their small size bacteria have by far the largest surface-to-volume ratio in the living world, which allows them to maximize their metabolic rates because of a high exchange of molecules and metabolites through their surface. The metabolic flexibility of microorganisms helps them to produce hundreds of different enzymes for all sorts of reactions. The areas of microbial biotechnology that are now receiving attention in the application of the newer concepts of genetic engineering of microorganisms for their improvement for carrying out biotransformation of substrates.

The Chemical alteration of a substance (substrate) as by the action of enzymes is called enzymatic biotransformation [Emily Monosson et al. 2007]. Currently there are a number of well-established chemical technologies for introducing chirality into a molecule. On the other hand, biotransformation will be useful in cases where it can save energy and elimination of some downstream process which are the key factor for cost strategy or it might allow extension of the bio-arsenal for transformations.

Fatty acids exist in nature as pure substances or as parts of more complex molecules known as lipids. The other, possibly preferable, definition states that lipids are compounds comprising fixed oils, fats, and waxes. However, this definition excludes the lipopolysaccharides (LPS), lipoproteins, glycerophospholipids, glycoglycerolipids and related compounds and the sphingo lipids, which are also undoubtedly lipids. The production of fatty acids by the hydrolysis (biotransformation) of natural oils and fats is a very important component in the economic exploitation of these naturally produced renewable raw materials. These products include oils from corn, rapeseed, sunflower, palm, coconut, olives and rice bran, and a wide range of animal fats such as tallow's. A significant number of high-value products require fatty acids in their manufactures. These include coatings, adhesives, specially lubricating oils, shampoos, baby food nutrients and other personal care products. Bacteria are intrinsically capable of synthesizing fatty acids, which are precursors in the biosynthesis of their cell envelopes.

Coconut (*Cocos nucifera*) (family Aracaceae) is mainly used as a staple food crop, and a source of wood and handicrafts, among many other uses, and is thought by many to be the world's most useful medicinal plant in tropical and subtropical countries [Chen & Elevitch, 2006]. For thousands of years, coconut oil products used in traditional medicine are believed to be antiblennorrhagic, antibronchitis, antifebrile and antigingivitic [Mandal & Mandal, 2011]. Coconut oil is very stable as it has a low oxidation point and the oxidation begins after 2 years of storage. This stability is due to the higher content of saturated fat. Moreover, this oil is rich in medium chain fatty acids and exhibits good digestibility [Che Man & Marina, 2006]. The three valuable medium chain fatty acids in coconut fat are (C12:0) lauric acid, (C10:0) capric acid and (C8:0) caprylic acid [Obi, Oyi, & Onaolapo, 2010]. Production of fatty acids and glycerol from fats and oils is important especially for oleochemical industries.

Vegetable fats are very important source of fatty acids which have high antimicrobial activity against bacteria, fungi, viruses and protozoa [Isaacs et. al. 1995; Ogbolu et. al. 2007; Ouattara et. al. 1997]. The current techniques for production of fatty acids are based on chemical and physical methods which operate at higher temperature and pressure [Bahruddin et.al.2007, Kent et. al. 1974]. The advantages of the microbial hydrolysis technique include the use of bio-route technology that operates at mild temperature, simple operational procedure, low cost as well as energy consumption [Destain et. al. 1997] and environment friendly. Microbial hydrolysis of coconut fat with active lipases and inactivation of harmful microorganisms by resulting fatty acids already have been studied for *Candida rugosa* and *Yarrowia lipolytica* [Isaacs et. al. 1991;Ting Tung et. al. 2006, Ota et. al. 1968].

Microbial lipases constitute an important group of biotechnologically valuable enzymes, mainly because of the versatility of their applied properties and ease of mass production [Macrae 1983; Sugiura, M. 1984; Godris H.L et. al. 1987; Copineau et. al. 1988; Suzuki et. al. 1988]. Most of the bacterial lipases are inducible extracellular enzymes. The extracellular enzymes are synthesized within the cell and exported to its external surface or environment[Fukumoto, J. et. al. 1963; Winkler, U. K. et. al. 1979; Ota, Y. et. al. 1982; Stuer W. et. al. 1986; Sugiura, M. 1984]. Lipases play an important role in the processing of γ-linolenic acid, a polyunsaturated fatty acid (PUFA); astaxanthine, a food colorant; methyl ketones, flavor molecules characteristic of blue cheese; 4-hydroxydecanoic acid used as a precursor of γ-decalactone, a fruit flavor; dicarboxylic acids for use as prepolymers; inter esterification of cheaper glycerides to more valuable forms (e.g., cocoa butter replacements for use in chocolate manufacture)[Undurraga et al., 2001] modification of vegetable oils at position 2 of the triglyceride, to obtain fats similar to human milk fat for use in baby feeds; lipid esters including isopropyl myristate, for use in cosmetics; and monoglycerides for use as emulsifiers in food and pharmaceutical applications.

Lauric acid is a saturated fatty acid with a 12 carbon atom chain thus falling into medium chain fatty acid [web ref. 1]. Lauric acid, as a component of triglycerides, comprises about half of the fatty acid content in coconut oil, laurel oil. The chemical name for lauric acid is dodecanoic acid. Due to its chemical composition, it is able to interact with polar solvent such as water. Lauric acid is able to bond with grease found in hair, and water can then be used to wash it away. When lauric acid is ingested, it is transformed into monolaurin which exhibits antiviral, antimicrobial, antiprotozoal and antifungal properties. It acts by disrupting the lipid membranes in organisms like fungus bacteria and virus, thus destroying them [web ref. 2]. Researchers in the Philippines have even begun studies to prove the effectiveness of lauric acid against HIV/AIDS because of its strong antiviral properties. Breast milk is the only other natural source that contains such a high concentration of lauric acid, which could explain the drastic decrease of infections of all types in breast feed babies with levels as high, is human breast milk (6.2%). Cow's milk also contains it, but it account for only 2.9% of the milk's total fat content and goat's milk contain 3.1% of its total fat[Beare-Rogers et. al. 2001].

To enhance the biotransformation process, the microbial population present in the system have to utilize the carbon source. It involves the addition of nutrients to increase the rate of biotransformation process. Inadequacy in nutrient concentration affects the biotransformation process. Hence, addition of appropriate nutrients in sufficient quantity is very essential to promote or enhance the microbial growth. For the optimization of n no. index entries found. Different methods were reported and Taguchi Method is one of the predominant techniques used recently. Optimization by Taguchi method using orthogonal array (OA) experimental design (DOE) involves the study of any given system by a set of independent variables (factors) over a specific region of interest (levels) [Tripathi and Srivastava, 2011]. This approach not only helps in extensive saving in time but also leads to more fully developed process by providing systematic, simple and efficient methodology for the optimization of the near optimum design parameters with only a few well defined experimental sets [Rao et al., 2008].

The selection process of process parameters like Temperature, pH, Substrate concentration and agitation (RPM) on microbial growth response towards the different concentration, so the ranking determines the combination of different parameters which yields the greater biomass and product (Lauric acid). The selection of the best combination of parameters in this traditional method is based on the response of microbial biomass and product concentration (Lauric acid) which belongs to multiple attributes decision problems. Grey relational analysis in the Grey theory is a simple and accurate method for multiple attributes decision problems [Tsai et al., 2003], especially for those problems with very unique characteristics. Therefore, this study will utilize the Grey relational analysis to establish a complete and accurate evaluation model for selecting appropriate combination of parameters. This methodology will yield higher microbial biomass with higher product concentration(Lauric acid) [Tsai et al., 2003].

Here we reported the use of statistical design of the experiments combining with Grey relational analysis (GRA) to reduce the total number of attempts for optimizing the process parameters, and we expected that this statistical tool will derive the best parameter (factor) combination for better growth of microbial biomass with higher product concentration. Statistical design of experiments determines the important effects of factors on a response as well as the interaction effects among the factors [Rao et al., 2008]. Although statistical experimental design with a combination of GRA has largely been employed in the optimization process parameters in grinding, turning operations and welding works in mechanical industry, it has been rarely applied to bioprocesses [Chung et al., 2008].

1.2 Research Objectives:

The main objective of my study is develop a technology for the production of industrially useful product from coconut oil by the biotransformation i.e Transformation of coconut oil to medium chain fatty acid(lauric acid) by the help of microbes/enzymes and comparison of lauric acid production under two different conditions(aerobic & anaerobic). The Specific objective of this study are as follows:

- To study the growth pattern of selected microbe in aerobic and anaerobic process by providing suitable condition.
- To study the process parameters for optimization of Biotransformation process with the help of Taguchi method by taking parameters as Temperature, pH, Substrate concentration and agitation (RPM).
- To study the production of lauric acid with the help of substrate and product kinetics by analyzing Gas chromatography-Mass spectrometry (GC-MS) method.
- To study the presence of enzyme and its activity, optimizations of process parameters for determining maximum enzyme activity like (pH, Temperature, Enzyme Concentration, Substrate Concentration & Time of Incubation).
- To isolate the gene which is responsible for biotransformation process by producing certain enzyme and transfer of gene into *E.coli* by means of making cloned product which helps in biotransformation process without maintaining the anaerobic condition.
- To study the antimicrobial activity of lauric acid against both gram positive and negative bacteria.

1.3 Scope of this study:

This study shall provide an alternative solution for production of fatty acid by means of biological method. It will provide an idea on how microorganisms present in the natural environment which have the ability to transform natural oil into fatty acid by producing certain enzyme. The novelty of this study would be the use of *Pseudomonas aeruginosa* in anaerobic condition for production of fatty acid. It will also emphasize the influence of process parameters of microbial enzyme on the process of biotransformation.

1.4 Thesis Summary:

This thesis comprises of five chapter's *viz.* Introduction, Literature Review, Materials and methods, Results and Discussion and Conclusion, each of detailed adequately to describe the methods used, research findings and outcome.

Chapter 1 Introduces the field of research in brief, comprehensive description of research background, research objectives and thesis overview.

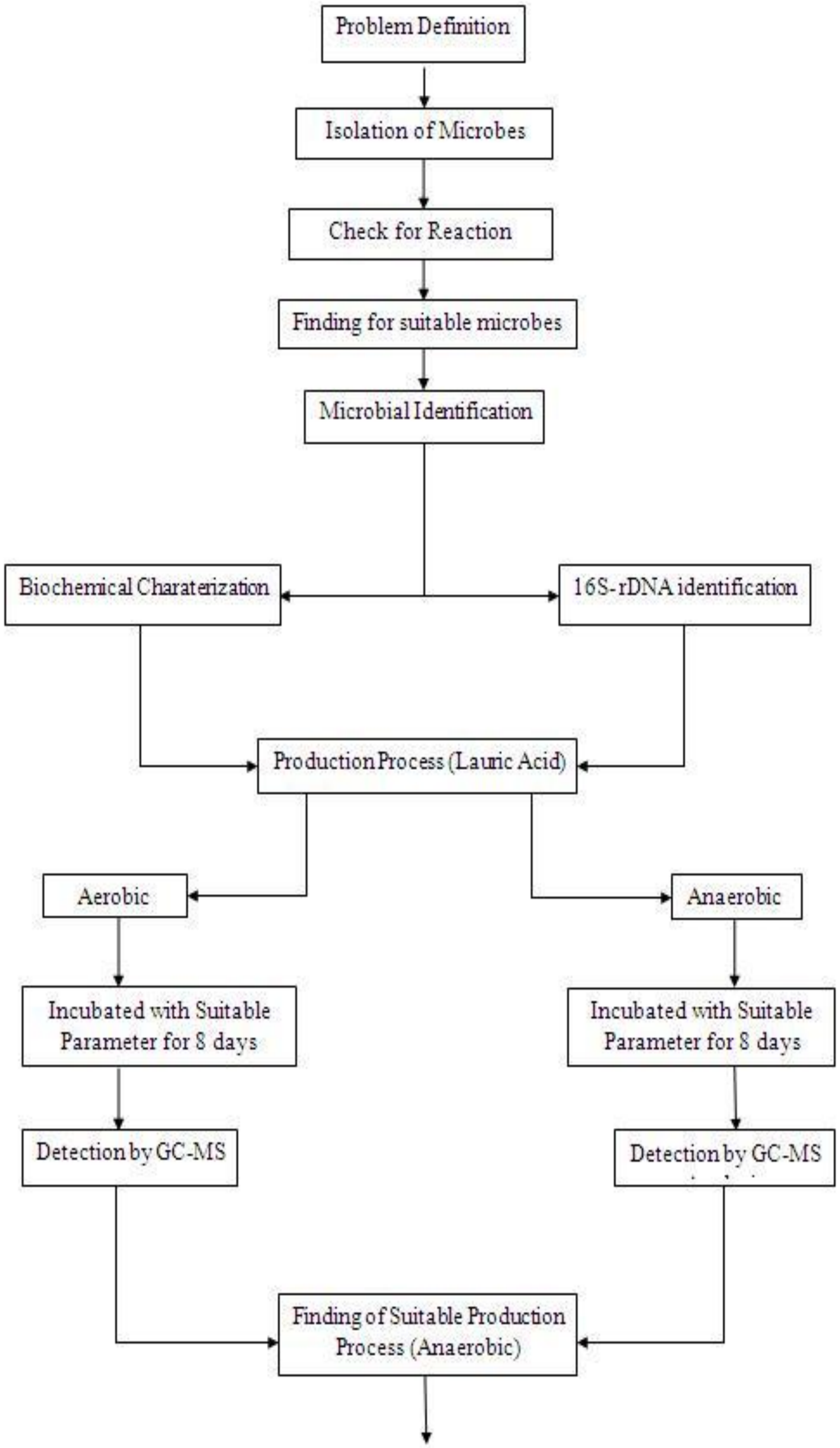
Chapter 2 discusses the literature reports in detail pertaining related to field of biotransformation and fatty acid production.

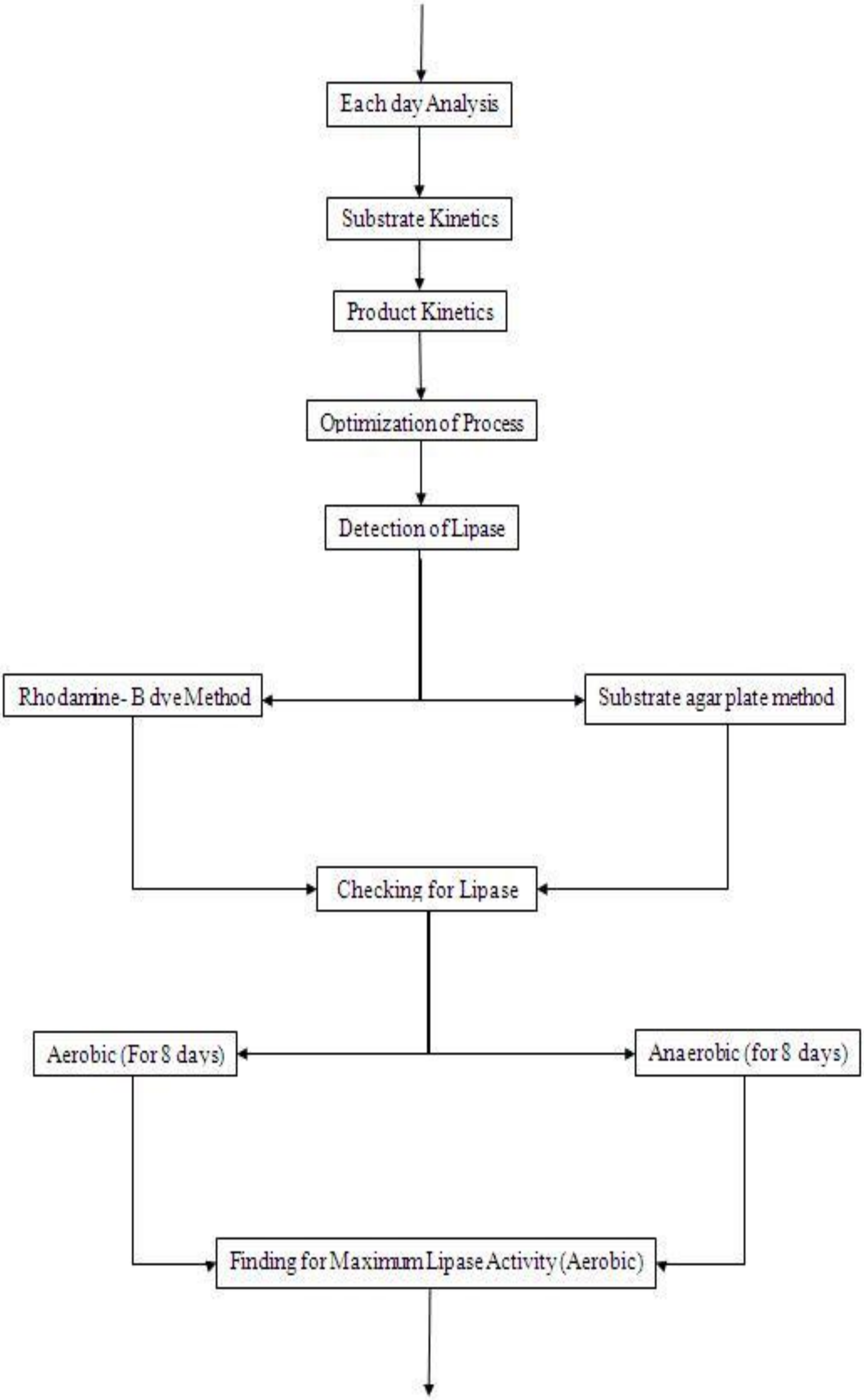
Chapter 3 describes the methodologies and protocols followed in biotransformation process for fatty acid production and enzymatic activity includes all the materials and methods involved in the work.

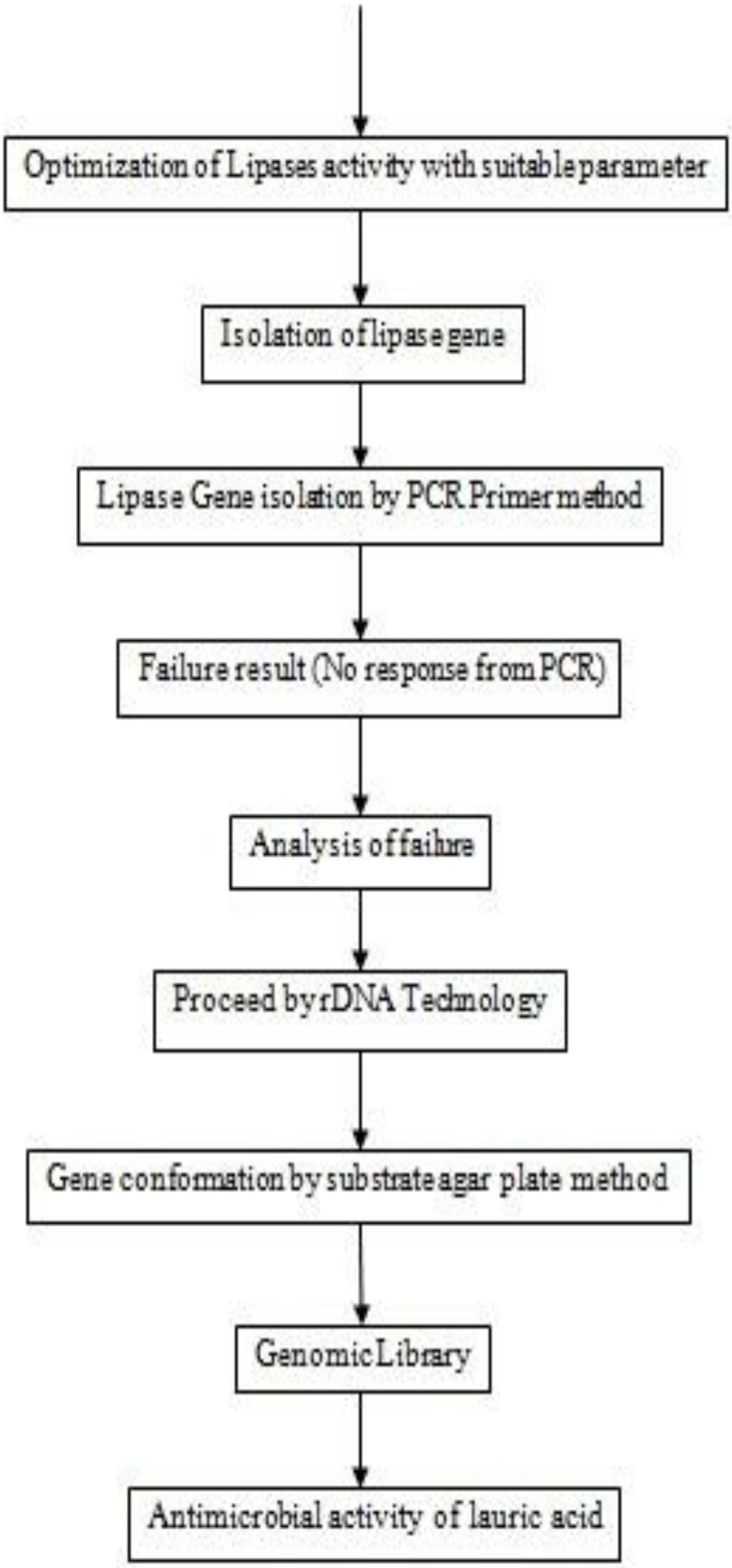
Chapter 4 provides the various results and observation found during this study and explains those with adequate reasoning and evidence.

Chapter 5 concludes the results and observation made during this study. Future recommendation based on the research outcome is suggested. The major highlights of this work are also summarized.

1.5 Work Process flow sheet:




Each day Analysis
Substrate Kinetics
Product Kinetics
Optimization of Process
Detection of Lipase
Rhodamine- B dye Method
Substrate agar plate method
Checking for Lipase
Aerobic (For 8 days)
Anaerobic (for 8 days)
Finding for Maximum Lipase Activity (Aerobic)


Optimization of Lipases activity with suitable parameter
Isolation of lipase gene
Lipase Gene isolation by PCR Primer method
Failure result (No response from PCR)
Analysis of failure
Proceed by rDNA Technology
Gene conformation by substrate agar plate method
Genomic Library
Antimicrobial activity of lauric acid

CHAPTER-2

LITERATURE REVIEW

This chapter is dedicated entirely to discuss in detail the reported works by various research groups in the field of biotransformation and particularly focusing on aspects of development of different techniques for fatty acid production. A comprehensive review of different method of transformation & its aspects, suitable substrate for transformation and condition for transformation is described in detail. The underlying principle of fatty acid production by biotransformation, role of enzymes and different methods related to this topic are highlighted. Further, important prospective applications reported in literature are summarized.

2.1 Overview of fatty acid:

Fatty acids, esterified to glycerol, are the main constituents of oils and fats. The industrial exploitation of oils and fats, both for food and oleochemical products, is based on chemical modification of both the carboxyl and unsaturated groups present in fatty acids. Fatty acids are almost entirely straight chain aliphatic carboxylic acids. The broadest definition includes all chain lengths but most natural fatty acids are C4 to C28 [IUPAC Terminology, 1997], with C18 most common. The chain is built from two carbon units, and cis double bonds are inserted by desaturase enzymes at specific positions relative to the carboxyl group. A large number of fatty acids varying in chain length. Fatty acids are carbon chains with a methyl group at one end of the molecule (designated omega, ω) and a carboxyl group at the other end. The carbon atom next to the carboxyl group is called the α carbon, and the subsequent one the β carbon. The nomenclature for fatty acid is:

$$\underset{\omega}{CH_3} - (CH_2)_n - \underset{\beta}{CH_2} - \underset{\alpha}{CH_2} - COOH$$

The letter n is also often used instead of the Greek ω to indicate the position of the double bond closest to the methyl end. The systematic nomenclature for fatty acids may also indicate the location of double bonds with reference to the carboxyl group. Fatty acids are usually derived from triglycerides or phospholipids. When they are not attached to other molecules, they are known as "free" fatty acids. Fatty acids are important sources of fuel because when metabolized they yield large quantities of ATP. Many cell types can use either glucose or fatty acids for this purpose. Fatty acid chains differ by length, often categorized as short to very long. Short-chain fatty acids (SCFA) are fatty acids with aliphatic tails of fewer than six carbons. Medium-chain fatty acids (MCFA) are fatty acids with aliphatic tails of 6–12 carbons, which can form medium-chain triglycerides. Long-chain fatty acids (LCFA) are fatty acids with aliphatic tails 13 to 21 carbons. Very long chain fatty acids (VLCFA) are fatty acids with aliphatic tails longer than 22 carbons.

Table 2.1: Fatty Acids in Commodity Oils and Fats [Gunstone et. al. 1994]

Fatty Acid	Common Name	Formula	Chain Length
4:0	Butyric	$CH_3(CH_2)_2CO_2H$	Short
6:0	Caproic	$CH_3(CH_2)_4CO_2H$	Short
8:0	Caprylic	$CH_3(CH_2)_6CO_2H$	Short/Medium
10:0	Capric	$CH_3(CH_2)_8CO_2H$	Medium
12:0	Lauric	$CH_3(CH_2)_{10}CO_2H$	Medium
14:0	Myristic acid	$CH_3(CH_2)_{12}CO_2H$	Long
16:0	Palmitic	$CH_3(CH_2)_{14}CO_2H$	Long
18:0	Stearic	$CH_3(CH_2)_{16}CO_2H$	Long
18:1	Oleic	$CH_3(CH_2)_7CH{=}CH(CH_2)_7CO_2H$	Long
18:2	Linoleic	$CH_3(CH_2)_4(CH{=}CHCH_2)_2(CH_2)_6CO_2H$	Long
18:3	α-linolenic	$CH_3CH_2(CH{=}CHCH_2)_3(CH_2)_6CO_2H$	Long
22:1	Erucic	$CH_3(CH_2)_7CH{=}CH(CH_2)_{11}CO_2H$	Long
20:5	EPA	$CH_3CH_2(CH{=}CHCH_2)_5(CH_2)_2CO_2H$	Long
22:6	DHA	$CH_3CH_2(CH{=}CHCH_2)_6CH_2CO_2H$	Long

2.1.1 Saturated Fatty acids:

Saturated fat consists of triglycerides containing only saturated fatty acids. Saturated fatty acids have no double bonds between the individual carbon atoms of the fatty acid chain. So, the chain of carbon atoms is fully saturated with hydrogen atoms. There are many kinds of naturally occurring saturated fatty acids, which differ mainly in number of carbon atoms, from 3 carbons (propionic acid) to 36 (hexatriacontanoic acid). Most saturated fatty acids are straight hydrocarbon chains with an even number of carbon atoms. The most common fatty acids contain 12–22 carbon atoms.Ssaturated fats are often thought of as a group there are many different saturated fats and each has different uses in the body and potentially different health effects. There is some evidence for positive effects of some saturated fats including antibacterial properties. Some saturated fats tend to raise both LDL and HDL cholesterol.

Table 2.2: Example of Saturated fatty acids [web ref. 3]

Common Name	Chemical Formula	Carbon Number
Caprylic acid	$CH_3(CH_2)_6COOH$	8:0
Capric acid	$CH_3(CH_2)_8COOH$	10:0
Lauric acid	$CH_3(CH_2)_{10}COOH$	12:0
Myristic acid	$CH_3(CH_2)_{12}COOH$	14:0
Palmitic acid	$CH_3(CH_2)_{14}COOH$	16:0
Stearic acid	$CH_3(CH_2)_{16}COOH$	18:0
Arachidic acid	$CH_3(CH_2)_{18}COOH$	20:0
Behenic acid	$CH_3(CH_2)_{20}COOH$	22:0
Lignoceric acid	$CH_3(CH_2)_{22}COOH$	24:0
Cerotic acid	$CH_3(CH_2)_{24}COOH$	26:0

2.1.2 Unsaturated Fatty acids:

Monounsaturated fatty acids have one carbon–carbon double bond which can occur in different positions. The most common have a chain length of 16–22 and a double bond with the cis configuration. This means that the hydrogen atoms on either side of the double bond are oriented in the same direction. The presence of a double bond causes restriction in the mobility of the acyl chain at that point. The cis configuration gives a kink in the molecular shape and cis fatty acids are thermodynamically less stable than the trans forms. The cis fatty acids have lower melting points than the trans fatty acids or their saturated counterparts. In polyunsaturated fatty acids (PUFAs) the first double bond may be found between the third and the fourth carbon atom from the ω carbon; these are called ω-3 fatty acids. If the first double bond is between the sixth and seventh carbon atom then they are called ω-6 fatty acids. The double bonds in PUFAs are separated from each other by a methylene grouping. PUFAs which are produced only by plants and phytoplankton are essential to all higher organisms including mammals and fish. ω-3 and ω-6 fatty acids cannot be interconverted, and both are essential nutrients. PUFAs are further metabolized in the body by the addition of carbon atoms and by desaturation. Mammals have desaturases (extraction of hydrogen)that are capable of removing hydrogens only from carbon atoms between an existing double bond and the carboxyl group β-oxidation of fatty acids may take place in either mitochondria or peroxisomes.The greater the degree of unsaturation in a fatty acid (i.e., the more double bonds in the fatty acid) the more vulnerable it is to lipid peroxidation (rancidity).

2.1.3 Medium Chain Fatty acid:

The medium-chain fatty acids (MCFA) are found in (the corresponding number of carbons) caproic acid(C6), caprylic acid(C8), capric acid(C10) and lauric acid(C12). It is found in oils and fats as triacylglycerol. Coconut oil and palm kernel oil are the oils used as foods with a higher content of lauric acid. Here we focused in production of lauric acid due to its versatile application and availability of substrate abundantly. Its main application is the phase change material in eutectic mixtures with other fatty acids [Tuncbilek K et. al., 2005]. Some other main applications of the lauric acid are listed below.

- Lauric acid is often used to investigate the molar mass of an unknown substance due to its melting point when pure is relatively high (43.2 °C).
- Lauric acid is used for treating viral infections including influenza (the flu); swine flu; avian flu; the common cold; fever blisters, cold sores, and genital herpes caused by herpes simplex virus (HSV). So it is used as medicine.
- Lauric acid is basically non-toxic, which gives it a distinct advantage over modern pharmaceutical drugs that are typically used to fight viruses, bacterial infections and fungal infections.
- Lauric acid is used as a vegetable shortening in food.
- Lauric acid is used to make detergents, soap, shampoo and cosmetics in manufacturing.
- Lauric acid is used to increase total cholesterol the most of all fatty acids but it has a more favorable effect on total HDL(High density lipoprotein) cholesterol than any other fatty acid, either saturated or unsaturated; a lower total/HDL cholesterol ratio suggests a decrease in atherosclerotic risk.
- Lauric acid is used for pretreatment of fibers in the textile, painting, varnish manufacturing industries and as surfactants in the petroleum industry [Gervajio G.C et. al. 2005].

Lauric acid is used in industries for different application. The total amount of lauric acid consumed in different industries with respect to their other raw materials is listed in table 2.2.

Table 2.3 Uses of Lauric acid (% Consumed) [web ref. 4]

Industry	Application	% of Lauric acid Consumed
Detergent & Soap	In manufacture of fatty esters used as surfactants	15
Food	Composition of baby food and production of cream	8-10
Renewable energy	In manufacture of methyl laurate used as biodiesel for blending	12-20
Cosmetics	Manufacture of Antiseptic cream and face cleaner	6-9
Pharmaceutical	Major Part in some medicine and syrup	5-15

2.2 Back ground of coconut oil (substrate):

Coconut oil is edible oil that has been consumed in tropical countries for thousands of years. Philippines, Indonesia, India, Sri Lanka, Mexico, West Malaysia, and Papua & New Guinea are the seven countries which produce major quantities of coconut in the world. Coconut is available in two forms viz., wet and dry materials commonly known as wet coconut and dry coconut or copra. The oil can be extracted from both these raw materials. In India and Srilanka it is a general practice to use only copra for oil extraction and the oil is used for food and cosmetic purposes. In some countries solvent extraction of the dry coconut followed by refining, bleaching and deodorization is carried out to get the refined bleached and deodorized coconut oil. The technology for the production of coconut oil through expellers is well developed and many medium scale industries in India produce oil by this method. However, some small scale industries produce the oil by processing fresh coconut also using local expeller press.

It has a long shelf life and is used in baking industries, processed foods, infant formulae, pharmaceuticals, cosmetics and as hair oil. Coconut oil has >90% saturated fatty acids; hence it is less attractive to consumers. Saturated fat is one that has no unsaturation or double bonds and tends to be solid at room temperature. It is rich in short and medium chain fatty acids. Various fractions of coconut oil are used as drugs. Butyric acid is used to treat cancer, while lauric acid is effective in treating viral infections.

2.2.1 Importance of coconut oil in India:

The coconut palm is the most important perennial source of oil which is grown in India. The cultivation of coconut is spread over the entire coastal belt and also some interior tracts. Compared to all other oil seed crops coconut has the highest productivity as well as consistency in production. Compared to other oil seed crops coconut is less susceptible to abnormal climatic condition. Indonesia and the Philippines are the first and the second largest coconut producing country in the world. India is the third largest coconut producing country. The major coconut growing states in India are Kerala, Tamilnadu, Karnataka, Andhra pradesh, West Bengal, Maharashtra, Orissa, Assam, Goa, Daman and Diu, Lakshadeep, Gujarat. Kerala tops in production accounting 39 percent of total production in the country.

Table 2.4: Coconut area and production in Asian and Pacific Coconut Community member countries [web ref. 5]

Country	Coconut area (hectares)	Coconut production	
		Million nuts	Copra equivalent (Tonnes)
Fiji Islands	60,000	150	25,000
India	1,903,000	14744	2,100.000
Indonesia	3,799,000	16,235	3,247,000
Kiribati	29,000	131	26,000
Malaysia	115,000	390	78,000
Marshall Islands	8,000	27.5	6,000
Philippines	3,380,000	12,573	2,399,000
Samoa	93,000	180	60,000
Sri Lanka	395,000	2,909	556,000
Thailand	247,000	1,186	356,000
Vanuatu	96,000	308	61,000
Vietnam	141,000	760.08	169,000

2.2.2 Specification for coconut oil:

Indian-Standard IS: 6220-1971 specifies the quality parameters of different grading for dry coconut uses in India. This standard prescribes the methods of grading and the requirements of dry coconut for extraction of oil and for regular use together with methods of sampling and test. The three types of copra are defined: type 1 (grades 1, 2 and 3), ball dry coconut for regular purpose; type 2 (grades 1 and 2), cup dry coconut for regular purpose; and type 3 (grades 1, 2 and 3), milling dry coconut for oil extraction. The material obtained from the fruits of *Cocos nucifera Linn*. Coconut oil is classified into 4 grades: refined, deodorized (neutralized, bleached, final steam deodorization); refined (no steam treatment); white oil (higher free fatty acids); and industrial oil (crude oil, not edible without processing).

Table 2.5: Indian specification for coconut oil [Ceylon std., 1968]

Characteristics	Expressed				Solvent-extracted		
	Refined Grade	**Grade 1A(Raw)**	**Grade 1B(Raw)**	**Grade 2(Raw)**	**Refined Grade**	**Semi Refined**	**Grade 1(Raw)**
Moisture impurities (max) (%)	0.1	0.25	0.25	0.25	0.1	0.25	1.0
Colour Lovibond Y+5R	2	4	11	30	2	10	30
Refractive index at 40^0C	1.4480 to 1.4490	1.4480 to 1.4490	1.4480 to 1.4490	1.4480 to 1.4490	1.4480 to 1.4490	1.4480 to 1.4490	1.4480 to 1.4490
Specific gravity at 30^0C/30^0C	0.915 to 0.920	0.915 to 0.920	0.915 to 0.920	0.915 to 0.920	0.915 to 0.920	0.915 to 0.920	0.915 to 0.920
Saponification value(min)	250	250	250	250	250	250	250
Iodine value (wijs)	7.5 to 10	7.5 to 10	7.5 to 10	7.5 to 10	7.5 to 10	7.5 to 10	8.0 to 1.3
Acid value (max)	0.5	2.0	6.0	8.0	0.5	1.0	1.0
Unsaponifable (max)	0.5	0.8	0.8	0.8	0.5	0.8	1.0
Polenske value (min)	13.0	13.0	13.0	-	13.0	-	-
Flash point ^{0}C (min)	-	-	-	-	225	100	90

There are basically three types of coconut oil available. A refined coconut oil is usually rather tasteless and odourless because it has been refined. It can usually withstand slightly higher cooking temperatures before reaching its smoke point. Refined coconut oils do not offer the same health benefits of a virgin, completely raw coconut oil but they are still excellent sources of most of the beneficial fatty acids (like MCTs) [web ref. 6]. Virgin coconut oil is the purest, most natural, least processed (chemically-changed) form and look nearly as clear as water. Virgin Coconut oil is unrefined (has not been changed with heat processes). It is known as fractionated coconut oil because the long-chain fatty acids in the heavier part of the oil are filtered away to leave behind the medium-chain fatty acids that are able to penetrate skin layers to be absorbed in the body but not stored[web ref. 7] Hydrogenated coconut oil is not the same as natural coconut oil. It is a process wherein "unsaturated" fatty acids are changed into more "saturated" fatty acids. Natural coconut oils are heated to high temperatures and bombarded with hydrogen atoms to make them less prone to spoilage [web ref. 8].

2.2.3 Physico-chemical characteristics of coconut oil:

Physical and chemical properties of an oil or fat are of critical importance in determining its application area. This is particularly true of the large quantity and variety of oils and fats used in various purposes. Oils and fats are intimate mixtures of liquid and solid phases consisting of triacylglycerols. These features make their properties of particular interest and have resulted in their use in a wide range of special applications. Some of the important parameters of coconut oil are described below.

2.2.3.1 Solubility:

Coconut oil is insoluble in water. The temperature above its melting point it is completely miscible with most of the non-hydroxylic solvents such as light petroleum, benzene, carbon tetrachloride etc. In alcohol, coconut oil is more soluble than most common fats and oils.

2.2.3.2 Chemical composition:

Coconut oil contains a high proportion of glycerides of medium chain fatty acids. The oil is highly stable towards atmospheric oxidation. The oil is characterized by a low iodine value, high saponification value and high saturated fatty acids content and is a liquid at room temperatures of 27°C.

2.2.3.3 Unsaponifiable matters:

All natural fats contain minor quantities of substance other than fatty acid glycerides. The unsaponified constituent is mostly sterols. The unsaponifiable constituent of coconut oil includes a small amount of tocopherols and phytosterols (Table 2.7).

Table 2.6: fatty acid composition of coconut oil and some other natural oil [Strayer 2006]

Natural Oil	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C20:0	C22:0	Others
Coconut	7.0	5.4	51.6	20.2	8.4	2.5	-	-	-
Palm Kernel	-	1.2	48.9	22.9	12.2	1.3	-	-	-
Sunflower	-	-	-	-	6.3	3.0	-	-	-
Ricebran	-	-	-	0.4	22.9	1.8	0.5	-	-
Safflower	-	-	-	0.3	11.9	2.3	-	-	-
Sesame	-	-	-	-	10.3	5.8	-	-	-
Ground nut	-	-	-	-	14.0	3.8	1.2	3.4	-
Palm	-	-	0.2	1.1	42.6	3.8	1.2	3.4	-
Olive	-	-	-	-	12.0	2.5	-	-	1.4
Soyabean	-	-	-	-	11.6	4.0	-	-	1.4
Grape seed	-	-	-	-	7.2	4.8	-	-	0.4
Linseed	-	-	-	-	7.1	2.0	-	-	0.4

2.2.3.4 Chemistry of fatty acids and triglycerides (medium chain):

Medium chain triglycerides (MCTs) are a class of lipids in which three saturated fats are bound to a glycerol backbone. It distinguishes from other triglycerides is the fact that each fat molecule is between six and twelve carbons in length [Babayan 1988]. MCTs are component of many foods with coconut and palm oils being the dietary sources [Heydnger et. al. 1996] with the highest concentration. MCTs have a different pattern of absorption and utilization than long chain triglycerides (LCTs) that make up 97% of dietary fats. For absorption of LCTs to occur, the fatty acid chains must be separated from the glycerol backbone by the lipase enzyme.

All fats and oils are composed of triglyceride molecules, which are triesters of glycerol and fatty acids. The fats upon hydrolysis yield fatty acids and glycerol. There are two methods of classifying fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids. The second method of classification is based on molecular size or length of the carbon chain in the fatty acid. The size of the fatty acid is extremely important because physiological effects of medium-chain fatty acids in coconut oil are distinctly different from the long-chain fatty acids [Furman et. al.]. Almost all of the medium-chain triglycerides used

Table 2.7: Physico-chemical Characteristics of coconut oil [Mansor et. al. 2012]

Characteristics	**Virgin coconut oil from wet coconut**	**Unrefined coconut oil from copra**	**Refined coconut oil**
Appearance	Colourless	Slight brownish	Colourless
Odour	Coconut smell	Coconut smell	Odourless
Melting point(^{0}C)	24	24	24
Moisture (%)	<0.1	<0.1	<0.1
Iodine value (cg12/g)	7.5-10	7.5-10	7.5-10
Peroxide value (meq. O2/kg)	0-1	0-1	0-1
Saponification value(mg KOH/g)	245-255	245-255	250-255
Phospholipids (%)	0.1	0.1	0.0
Unsaponifiable matter (%)	-	0.42	0.19
Tocopherols (mg/kg)	150-200	150-200	4-100
Phytosterols (mg/kg)	-	400-1200	-
Saturated Fatty acids (%)	92.0	92.0	92.0
Monosaturated fatty acids (%)	6.0	6.0	6.0
Polyunsaturaetd fatty acids (%)	2.0	2.0	2.0

in research, medicine, and food products come from coconut oil. MCTs are not only found in coconut oil but also are natural and vital components of human breast milk. MCT are considered essential nutrients for infants as well as for people with serious digestive problems like cystic fibrosis [Johnson et. al. 1986, Marie-Pierre et. al 2003].

Banzon and Resurreccion[Claudio et. al. 1968] carried out a study on the fatty acid distribution in coconut oil obtained by different processing methods There was no observed change in the fatty acid distribution in samples of coconut oil obtained by different methods. Medium chain triacylglycerols are unique categories of lipids produced by the esterification of glycerol with medium chain fatty acids, which come from high lauric oils. Coconut and palm kernel oils are the only commercially important sources of medium chain fatty acids [Babayan 1968]. These oils are hydrolyzed to liberate their fatty acids from glycerol and then the fatty acids are separated by fractional distillation. The lower boiling or top fraction of the fatty acids contains the medium chain acids [Nandi et. al. 2005]. The esterification reaction between glycerol and the medium chain fatty acids is carried out at high temperatures with or without use of a catalyst. The water liberated in the reaction is removed continuously to drive the reaction to completion [Garfinkel et.al. 1992]. When the esterification reaction is complete, excess fatty acids are removed from the reaction mixture by vacuum distillation. The detailed method for different fatty acid production by chemical method is described below.

2.3 Processing for fatty acid by chemical method (Industrially):

Chemical refining is the common methods of crude coconut oil refining in most parts of the world. It involves the removal of free fatty acids and other impurities by means of chemical agents such as sodium hydroxide. This process causes considerable loss of oil in the emulsion and in adequate separation by centrifugation as well as pollution problems due to salt remaining after acidification of the soap stock to recover fatty acids and other volatiles and odours by stripping with in a single unit. Free fatty acids can be recovered however pre-treatment is required in the physical refining process to remove some impurities.

Palm kernel and coconut oils are particularly important to the fatty acid industry because they are the major sources of medium chain fatty acid. These in turn may be fractionated into saturated/unsaturated acids and to specific chain lengths by winterization, panning and pressing, fractional distillation, solvent crystallization and hydrophilization methods. The products are important raw materials for the soap, detergent and oleochemical industries. Coconut oil and palm oil are one of the significant part of the economies of the Asian countries. These oils are also very important in the world's supply of vegetable oils. Coconut oil and palm kernel oil are particularly significant to the fatty acid industry and its customers because they are the largest of a very few commercially available sources of lauric acid. These oils represent major raw materials for the manufacture of fatty acids and glycerine. These materials may lead to different desired processing steps to yield economically derived products for specific end-use applications. Some of Important steps for industrially production of fatty acids are

- Hydrloysis
- Distillation(Batch, continuous, Fractional & Molecular distillation)
- Separation
- Hydrogenation
- Methanolysis

2.3.1 Hydrolysis:

Hydrolysis usually means the cleavage of chemical bonds by the addition of water. Generally hydrolysis is a step where the degradation of a substance occurs. Hydrolysis usually requires the use of an acid or base catalyst and is used in the synthesis of many useful compounds. The first important step in processing these oils is splitting or hydrolysis of the triglyceride to yield glycerine and mixture of fatty acids. The hydrolysis reaction can be done batch wise according to the Twitchell process [Sonntag 1979] or continuously at high temperature and pressure using the Colgate-Emery process. This continuous counter current method takes from one to three hours to accomplish a 99% conversion. Today almost all fatty acid manufacturers use the continuous process as shown in figure 2.1. Different catalyst can be effectively used to split these oils; water through multiple effect evaporators, followed by distillation or deionization [Miner C.S. 1953, Newman A.A. 1968].

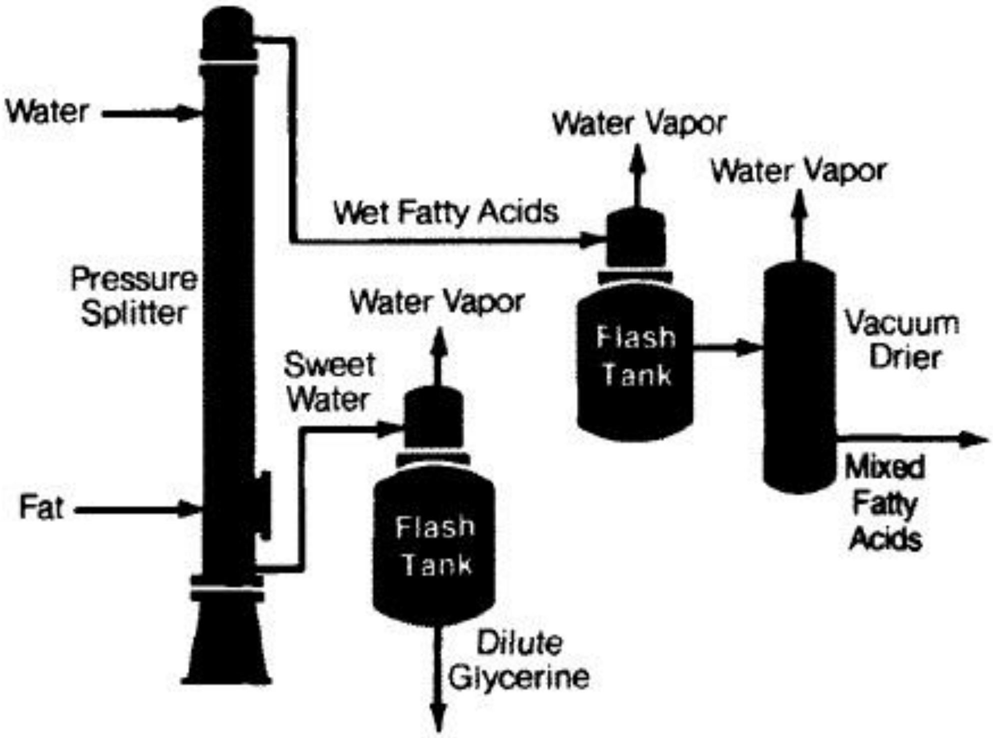


Figure 2.1: *Colgate-Emery hydrolysis process [Kent et. al. 1974]*

Glycerine is also a by-product of the methanolysis of these oils. Since both oils are of vegetable origin the glycerine can be processed to a certified kosher product. The natural glycerine from these oils can be processed by multiple refining techniques to a very high purity product which is equivalent to or purer than, glycerine derived synthetically.

2.3.2 Distillation:

Purification of fatty acids by distillation has been practiced for well over a hundred years and is still the most common and producing purify fatty acids. Distillation removes both the low and high boiling impurities as well as odor substances. Distillation of fatty acids may be either batch or continous process, at atmospheric pressure or under reduced pressure. It may be simple distillation involving purification of mixed fatty acids or fractional distillation consisting of both purification and separation of fatty acids according to chain length [Gervajio 2005, Muckerheide 1952, Potts R.H et. al.1953).The inherent sensitivity of fatty acids toward heat, the distillation methods employed should be conducted at as low a temperature as practically and economically feasible while maintaining the shortest residence time of the fatty acid in the distillation unit. Modern distillation units rely upon high vacuum, effective heating, short contact times, effective mass transfer between vapor and condensate, and steam economy [Lausberg et al. 2008].

The split fatty acid mixture may be refined by using distillation techniques. Distilled products normally represent the first salable fatty acid products from the processing of the oils. The resulting distillation product typically retains the fatty acid chain length distribution found in the original oil. One of the largest uses of this whole fatty acid is in the manufacture of soaps and shampoos. The wide range of chain length distribution is desired for such soap properties as flash foaming and bubble size. These acids may also be reacted at the carboxyl group to yield esters, alkanolamides, etc., which find utility in many end use applications.

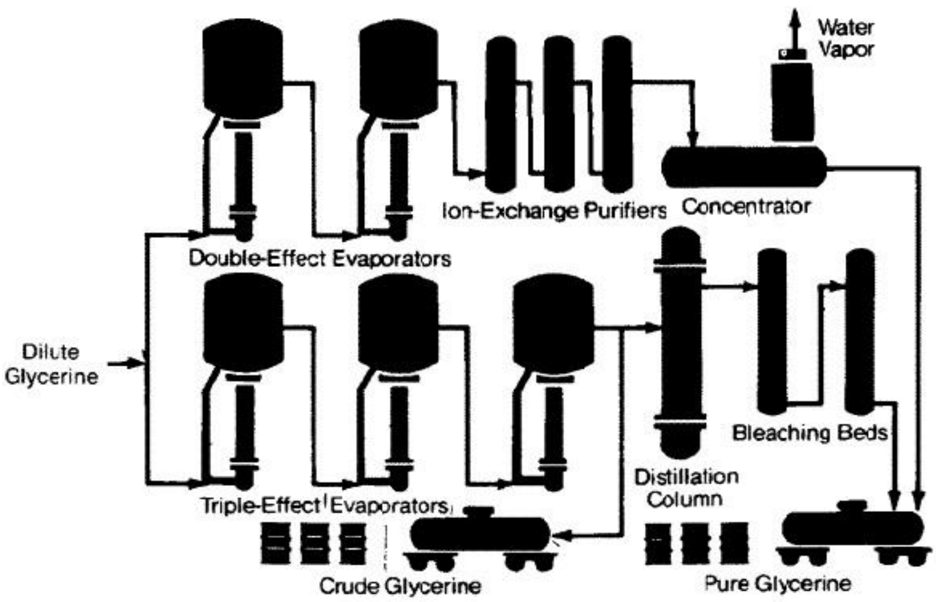


Figure 2.2: *Glycerine processing by multiple effect evaporator and distillation column [combs. 1985]*

2.3.2.1 Batch Distillation:

Batch distillation at atmospheric pressure is the commercial processes used in fatty acid distillation. It uses a direct-fired still pot fitted with a steam sparger. The pot is charged with fatty acids and heated to 260° to 316°C and sparged with saturated steam at 149°C. The ratio of steam to fatty acid vapor is typically 5 to 1. The steam and fatty acid vapor are condensed separately. The economics of this type of distillation is poor due to the large amount of steam used. Considerable amounts of fatty acids are also entrained in the steam condensate.

Distillation is further complicated because of the prolonged heating of the fatty acids at high temperatures and the inherent thermal instability of the fatty acids. This combination often results in considerable decarboxylation and polymerization with consequently large amounts of viscous residue and pitch. Fatty acids of about 95% hydrolysis when distilled in this manner yield 15 to 20% entrained fatty acids and 10 to 15% residue. Re-splitting the residue and distillation yields low quality fatty acids and a final pitch residue of 5 to 8% [Muckerheide 1952]. The improvements in this distillation technique included working at reduced pressure (5-50 mm Hg) and lowering the amount of injected steam. The water from the steam is desirable as it suppresses anhydride formation [Potts 1956].

2.3.2.2 Continous Distillation:

The first fatty acid continuous distillation was developed by Wecker at 1927. Preheated fatty acid feed enters through pipe and flows through a series of reaction chambers a interconnected by another pipe. The reaction chambers are heated at the bottom by gas or oil burners. Superheated steam is introduced through pipe and injected into the feed in each chamber by a sparger. The low pressure imposed in the reaction chamber and the high temperature of the feed caused the superheated steam to evaporate vigorously resulting in an instantaneous distillation of the fatty acids. The vapors are led to a pipe header condensed by a water cooled condenser and collected in another chamber. The steam passes on to the barometric condenser and the non-condensable gases are removed by a vacuum pump. The residue leaving the last reaction chamber is cooled and collected. Vacuum pressure maintained at 30-35 mm Hg and the temperature in chambers ranged from 196° to 260°F.

The disadvantage of steam distillation of fatty acids is the formation of emulsions in the last stage of condensation due to water spray. The calcium and magnesium salts in the water spray react with the fatty acids forming soaps. To recover the fatty acids, the soap is acidified and redistilled if desired. This can be avoided by employing dry distillation, i.e., distillation without using steam or any gaseous medium as carrier of the fatty acids. Such process was developed by Mills in 1942, employed a combination of dry and flash distillation to recover fatty acids from hydrolyzed fats and oils. The fatty acid which has to be distilled rapidly,heated using a heat exchanger to the boiling point corresponding to the operating pressure (≤ 12.7 mm Hg absolute). When the heated feed is introduced to the bottom of the tube and exposed to the lower pressure in the still, the fatty acids vaporizes immediately. The vapors lift the undistilled residue from the bottom of the still up the tube and splashes against the bottom of the baffle creating a continuous curtain of liquid undistilled material. The vapor proceeds to the condensers and the fatty acid condensates are collected in the receivers which can be collected continuously or intermittently. The undistilled material is collected continuously through pipe which can be directed by valve back to the heat exchanger or by valve to the residue collector.

2.3.2.3 Fractional Distillation:

Fatty acids are derived from natural sources; their initial and distilled compositional mixtures tend to vary even when the same type of fat or oil is used. Fractional distillation separates fatty acids based on their boiling points. Fatty acids which differ in chain length by two carbons are easily separated, thus; fatty acid fractions of 90% or better purity are obtained [Potts et. al. 1953, Ruston 1952]. Fundamentally fractional distillation is carried out in the same manner as continuous distillation. The main difference is in the design of the main fractionating column which is fitted with several bubble cap trays means for removal of side stream distillates of fatty acids and return part of these streams as reflux [Muckerheide 1952, Stage 1984]. In the fractionating column, vapors move upwards through the column and condensed at the top. Under ideal conditions, the heat lost by the rising vapor is gained by the descending condensate, with no heat loss or gain from the outside. The net result is the concentration of more volatile fractions on top of the column and the increasing concentration of less volatile fraction at the bottom of the column [Norris et. al. 1945].

Fractionating stills are custom designed to suit the feedstock and product requirements. With lauric type fatty acids from coconut and palm kernel oils, up to 30 fractionating trays can be used for highest purity fraction because of the higher volatility and greater stability of the shorter chain fatty acids. Long chain fatty acids like erucic (C22:1) in rapeseed oil have much lower vapor pressure and would need a limited number of fractionating trays to keep the reboiler below the decomposition temperature[Berger et. al. 1979]. Based on the compositions of the two oils, coconut oil generally yields a larger quantity of lauric acid and myristic acid. Many of these high-purity short-chain acids are reacted with mono and polyhydric alcohols to make esters that are formulated into synthetic lubricants. The myristic and palmitic acids may be reacted with isopropyl alcohol to make the corresponding esters for use as emotlients in cosmetic and personal care products. The lauric, myristic and palmitic acids may be converted to alcohols for use in detergent formulations. Fractional distillation will not efficiently separate the saturated stearic acid (C18) from the unsaturated oleic acid (C18) due to their boiling points and therefore other separation techniques are used for their separation[Berger R. et. al. 1979].

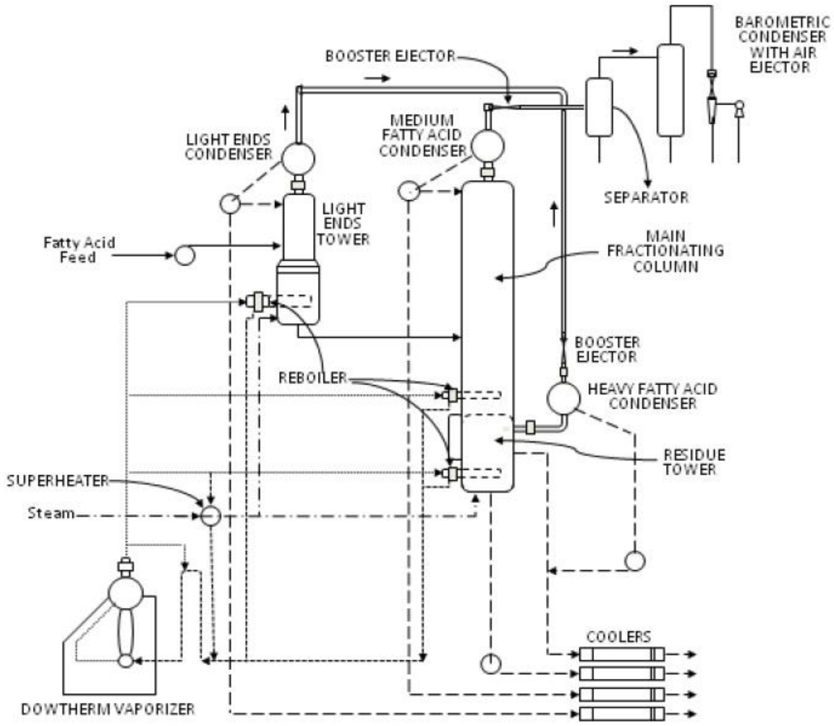


Figure 2.3: *Flow diagram of fractional distillation [Potts et. al. 1953]*

2.3.2.4 Molecular Distillation:

Molecular distillation is industrially useful in the purification of unstable or highly oxidatively unstable fats, oils and their derivatives. Molecular distillations are conducted under vacuum conditions, which allows for reduced distillation temperatures compared to conventional distillation techniques thus reducing the risk of oxidative damage. Current industrial applications include cosmetic applications, the concentration of omega-3 fatty acids (EPA and DHA) and corresponding esters in fish oil [Rossi et al., 2011] and contaminant removal. Additionally Vitamin E [Pramparo et al., 2005], Vitamin A, cocoa butter, dimer acids, epoxy resins, lubricants, monoglycerides, insecticides, pharmaceuticals, perfumery and flavours, essential oils, Azadirachtin (Neem based pesticides) and its formulations as well as many other natural & herbal products have been distilled on an industrial scale using this process. Molecular distillation consists of wiped film molecular distillation unit which is shown in fig.2.4.

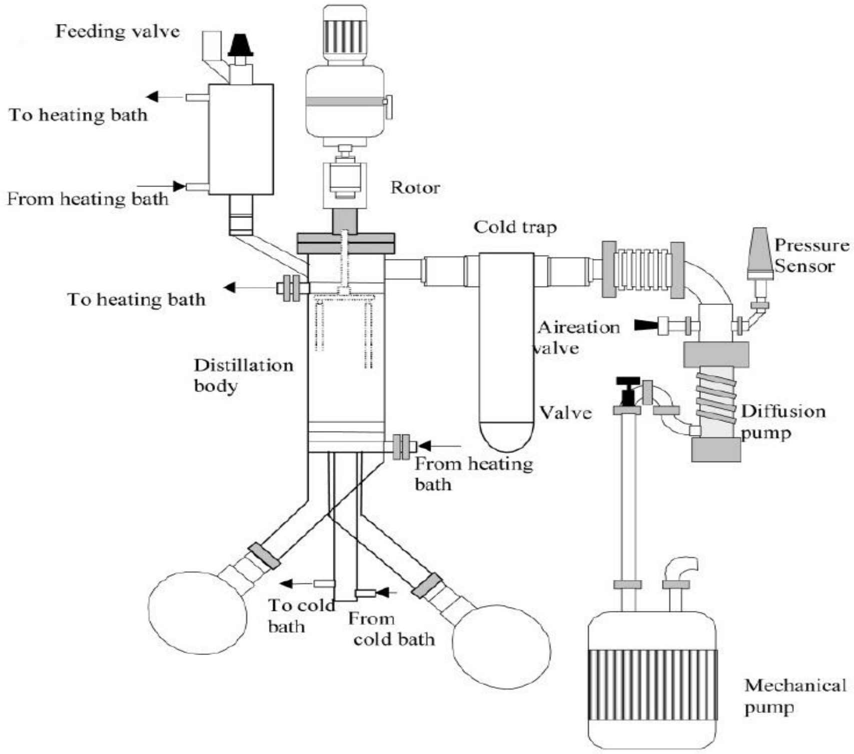


Figure 2.4: *Wiped film molecular distillation unit [Marttinello et al., 2008]*

2.3.3 Separation:-

Presently there are three commercial methods are used for separating saturated acids; winterization, panning & pressing process and the solvent crystallization process. The earliest method of physical separation of fatty acids, dating back to the late 1800's was called winterization or fractionation and effected a separation by the gradual cooling and crystallizing of the saturated acids. In the panning and pressing process, fatty acid mixtures were melted and poured into flat bottom pans and placed in a cool room to crystallize. The solvent is distilled from each fraction and recycled, while the resulting saturated and unsaturated acids are submitted to other refining operations. The Solexol process uses propane under pressure and the relative solubilities of the acids in propane to effect a separation.

Several newer methods of separation that are investigated include. They are selective adsorption, air entrainment, transesterification-fractional distillation, methyl formate solvent process and lithium soap separation. Due to their low oleic acid content, coconut acids are not normally solvent separated but rather are distilled and fractionated. Palm kernel oil has greater oleic acid content and therefore solvent separation may be of value. A combination of fractional distillation to remove the shorter chain length material (through C14) followed by solvent separation of the palm kernel fatty acids could also be used to obtain oleic acid. In many cases, oleic is combined with coconut or palm kernel fatty acids to provide differing desired foam structures and stabilities.

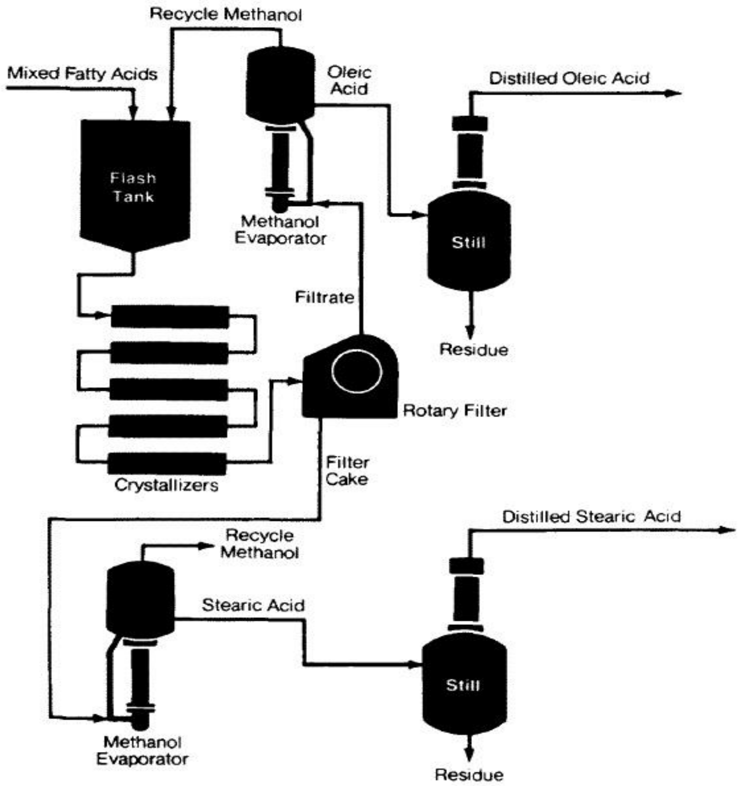


Figure 2.5: *The Emersol process of separation*

2.3.4 Hydrogenation:

Hydrogenation is referred to as hardening, is used to convert unsaturated fatty acids to saturated fatty acids. Often the fatty acids are refined by distillation prior to hydrogenation to remove sulfur contaminants and other materials detrimental to hydrogenation. The commercial hydrogenation of fatty acids has grown since the 1930's, when it was realized as an alternate approach to making a saturated product somewhat similar to the pressed or solvent separated acids. The main goal of hydrogenation is to lower the iodine value as quickly as possible. The efficiency of the catalyst and purity of the hydrogen are important factors in modern hydrogenation. Most fatty acids are hydrogenated in batch types of operation and utilize a catalyst such as nickel, platinum or palladium [Hastert 1979] with nickel being the most common and least expensive catalyst used in hydrogenation.

2.3.5 Methanolysis:

The whole oils reacted with alcohols in the presence of an alkaline catalyst to yield fatty acid esters and released recoverable glycerine. Methyl esters produced via the alcoholysis process or the direct reaction of the acid and alcohol may be distilled. These methyl esters find use in the manufacture of alkanolamides for emulsifiers and detergents, shampoos and general purpose cleaners, and transesterification to higher esters, e.g., isopropyl myristate and palmitate.

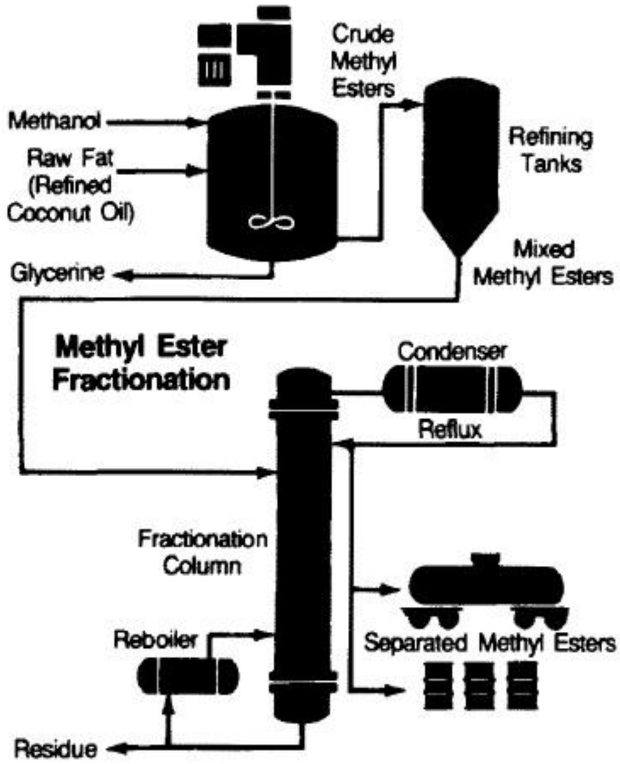


Figure 2.6: *Methanolysis Process*

The industrial chemistry of oils and fats is a mature technology with decades of experience and refinement behind current practices. Environmental pressures demand cleaner processes, and there is a market for new products. Current developments are in three areas ‘‘green’’ chemistry, using cleaner processes, less energy, and renewable resources; Microbe or enzyme catalyzed reactions, used both as environmentally friendly processes and to produce tailor-made products and novel chemistry to functionalize the carbon chain, leading to new compounds. Biotransformation is today considered to be an economically competitive and alternative technology in search of new production routes for fine chemical, pharmaceutical and agrochemical compounds [Davis et. al. 2001]. The detailed literature for biotransformation process is described below.

2.4 Biotransformation:

Biotransformation or biocatalysis encompasses the use of biological systems to catalyze the conversion of one compound to another. The catalyst part of the biological system consist of whole cells, cellular extracts, or isolated enzyme(s). The second decade of the twentieth century, processes based on biotransformation's were classified under various denominations such as ''zymotechnology'' or ''technical biology''. The concept of a more sustainable use of the limited resources was thus one of the driving forces for the rapid development of industrial biotechnology long before the now worldwide accepted political vision of sustainable development. Worldwide, interest in industrial biotechnology grows is a new technology that prove product capabilities and economics [David gaskin 2009]. Biotransformation processes have been used by mankind for several thousand years. For example the biotransformation of ethanol to acetic acid (vinegar) by *Acetobacter* was most likely developed concomitantly with ethanol production from fermentable sugars by our ancestors in Babylon (Mesopotamia). As a matter of fact, the biotransformation of ethanol to vinegar was probably also the first true biotransformation process applied in an industrial manner. Biotransformation is the process whereby a substance is changed from one chemical to another (transformed) by a reaction mediated by microorganisms or enzymes [National library of Medicine (NLM)] (Or) Biotransformation is defined as the use of biological systems to perform chemical changes on compounds that are not their natural substrates. The biological system could be the plant, animal, microorganisms or enzymes derived from the microorganisms [Jacquelyn 1999]. The use of living organisms to modify substances (substrates) that are not normally used for growth of microbes is called as microbial transformation [A. Schmid et al. 2001]. Also there is other approach of biotransformation called enzymatic biotransformation.

There are two reasons, that encourages to use biotransformation process include:

- The ability of microorganisms, e.g., bacteria, to produce large amounts of biomass and a great variety of different enzymes in a short time.
- The chemo-, regio-, and enantio selectivity of microbes or enzymes.

Biotransformations are chemical reactions that are catalyzed by microorganism in terms growing or resting cells or that are catalyzed by isolated enzymes. Because of the high

Stereo and regio-selectivity combined with high product purity and high enantiomeric excesses, biotransformation can be technically superior to traditional chemical synthesis. In current scenario there are a number of chemical methods available for transformation of various products but on the other side biotransformation is useful in the case where it can save energy and elimination of some downstream process which can affect the cost strategy of a product. Biocatalysis has matured to a standard technology in the fine chemicals industry, which is reflected by the number of biotransformation processes running on a commercial scale. Enzymes from all kinds of biological sources can now be recloned and overexpressed in easily mass cultivatable microorganism or cell cultures. With this technology even enzymes that have been rare up to the present can be produced in large quantities and at affordable costs. In addition, whole-cell biocatalysts or production organisms can now be genetically improved by directed engineering of metabolic pathways. According to OECD-report the state of the art and the future development needs biotransformation for industrial biotechnology [OECD 1998]. Some important conclusions in this report summarizing the state of the art are the following:

- Economic competitiveness has been established for a variety of biotransformational applications to achieve cleanliness.
- Industrial penetration of biotechnology is increasing as a consequence of advances in recombinant DNA technologies.
- Biotransformation operations have led to cleaner processes with lowered production of wastes and, in some cases, lower energy consumption.
- The fine chemicals industry is one of the industrial segments where the impact of biotransformation is felt most strongly.

It is clear that the choice between a biotransformation and chemical technology will be driven by the commercial performance of the selected synthetic strategy on a specific target. With the right cultivation conditions, microorganisms grow exponentially. Most microorganisms are also able to grow under varying conditions and on a great variety of substrates. However, these enzymes are not naturally over-produced but are regulated according to the physiological needs of the cells. Useful transformations may be simple one-step reactions or those involving a few sequential steps, where reaction products can be isolated and identified.

Advantages of Biotransformation:

- The ability of microorganisms, e.g.,bacteria, to produce large amounts of biomass and rate of multiplication of cell is more.
- Due to their small size, bacteria have by far the largest surface- to-volume ratio in the living world, which allows them to maximize their metabolic rates because of a high exchange of molecules and metabolites through their surface.
- Transformation reaction operates at near neutral pH, ambient temperatures and minimum energy utilization.
- Umpolung type reactions can be carried out.
- Microorganisms have great potential for inducing new or novel enzyme systems capable of converting foreign substrates.
- A combination of microbial, enzymatic and chemical transformations (chemo-enzymatic synthesis) can also be exploited.

Disadvantages of Biotransformation:

- Sometimes negative regulation (inhibitory and toxic properties of reactants or products) of biocatalyst.
- Biocatalysts are sensitive to environmental extremes of temperature, pH etc.
- Due to inhibition effect sometimes dilution of products occurs.
- Industrial inertia to change to new technology.

2.4.1 Challenges in Biotransformation:

As a customer manufacturing organisation (CMO) dealing mostly with a broad spectrum of organic molecules. These organic molecules vary from very flat and small to very large structures in which the synthetic complexity can be increased by the presence of many chiral centres. The main challenge faced by a CMO is to deliver the necessary quantity and quality of a key intermediate and with a cost-competitive process throughout the life cycle of the product. Therefore the activities of a process development should aim towards the development of a commercial process where manufacturability, robustness, and best fit to the plant will be established (Figure 2.7). Biotransformation is often perceived as the last option when the process chemist sees no other alternative. Nevertheless, depending on the business situation of the new chemical entity, such change might be accepted but often as a 2nd generation process significant cost improvement can be expected. For low volumes (early

Figure 2.7: *R & D Challenge to build a profitable business in Biotransformation process [Meyer et. al. 2005]*

phase requirements 10 to 100 kg) the development of a biotransformation route is too time-consuming compared to organic chemistry, especially if speed is requested for delivering material for different purposes.

2.4.2 Burning issues in Biotransformation:

The various burning issues in biotransformation process have been found from various literatures. Some of them are summarised below:

- The Availability of strains and enzymes, stable strains or enzymes from the different enzyme classes ready for use in a screening for a new product. A broad strain library is a key success factor for the biotransformation industry and innovation should focus on this aspect.
- Biotransformation "environment" is of second priority. Although, for most problems more or less practicable solutions are available today but there remain exceptions: (i) Product inhibition still waits for a scalable technical multiproduct solution to remove the inhibiting product from a biotransformation broth (ISPR, In Situ Product Removal). (ii) Solubility of reactants requiring non-aqueous reaction media.
- Down Stream Processing (DSP) is the least problematic aspect of biotransformation, as chemical processing technology for small molecules is available.

The lack of a broad strain and enzyme base is the factor most seriously impeding biotransformation. To overcome the strain shortage, creative technical as well as unusual nontechnical solutions are needed. The Swiss Industrial Biocatalysis Consortium has taken step to establish an unconventional solution by screening active strain and enzyme for use in commercial purposes [Meyer et. al. 2005].

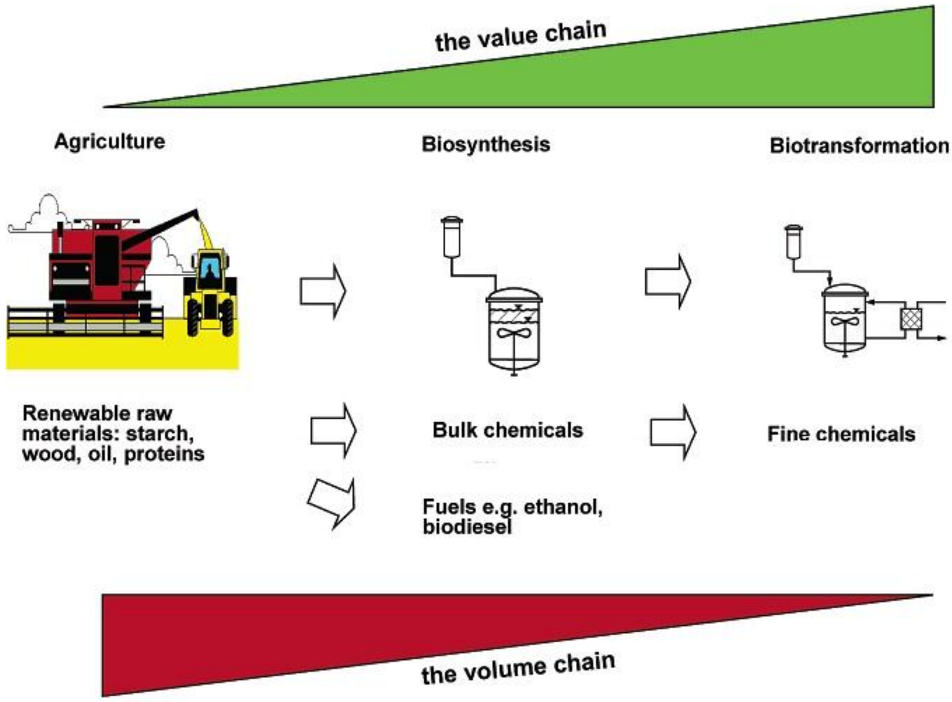


Figure 2.8: *The value and volume chain of Biotransformation [James et. al. 2006]*

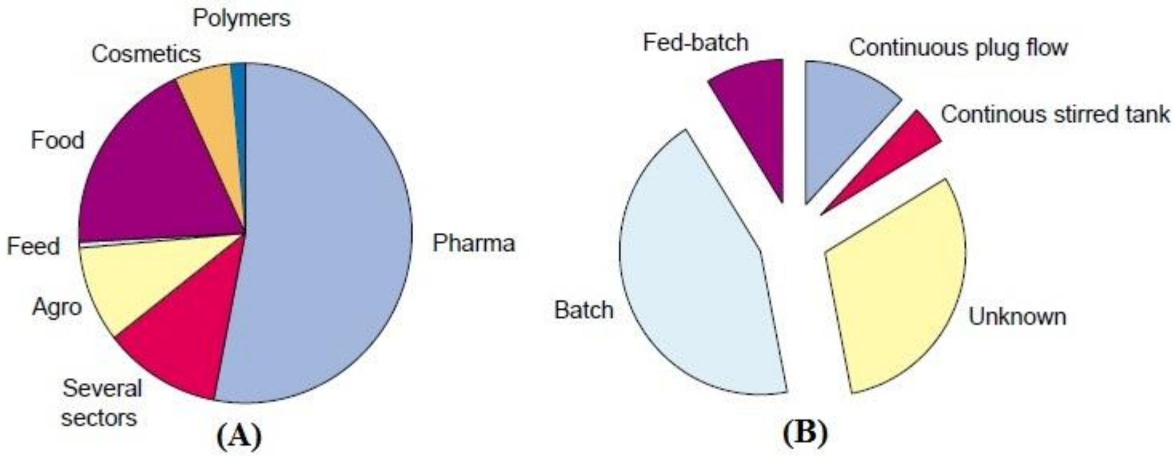


Figure 2.9: *(A) Industrial sectors in which the products of industrial biotransformation are used & (B) Reactor types used in industrial biotransformation [Current opinion in Biotechnology]*

2.4.3 Different techniques for Biotransformation process:

There is a growing interest in sustainable solutions for industrial manufacturing to replace non-renewable fossil fuel based chemical feedstocks in the long term. The various advanced techniques for biotransformation process have been found from various literatures. Some of them are summarised below:

2.4.3.1 Metagenomics:

Nothing can replace the need of finding and identifying new strains or novel enzyme activities. Unfortunately the standard microbiological cultivation and screening methods cannot deliver the wealth of novel strains and enzyme activities needed in an appropriate time frame. A common estimate among microbiologists is that the vast majority (99%) of prokaryotes has never been investigated in the laboratory and that their characteristics and potential are unknown. Whether these organisms are unculturable or whether they just do not grow on the media and culture conditions used, this enormous untapped reservoir of potentially new enzyme activities could not be used. Metagenomic gene discovery is extracting genes directly from nucleic acids gathered in the environment; bypassing tedious microbiological cultivation and product [Cowan et. al. 2005] by the potential of this direct mining of genes and novel enzymes. The inaccessible biospheres will become accessible; an example is the exploitation of the microbial habitat of deep subsurface biospheres [Teske 2005]. Here in our study we have isolated microorganism from three different places and selected a suitable microbe which can able to perform biotransformation to achieve our target product.

2.4.3.2 Enzyme evolution:

While the metagenomic approach is searching for novel microbial strain or enzyme in the natural reservoir, directed evolution is a different approach to reach the same goal. With directed evolution, an existing and known enzyme is going through repeated cycles of variations and screening to finally select an enzyme protein with the desired functions. Directed evolution combines techniques such as different genetic engineering methods to create genetic diversity, high throughput screening, advanced analytical and computational methods. In contrast to the classical and linear enzyme improvement, directed evolution is a branched process which allows the fast creation of novel enzyme variants for industrial application. As the functional recombinant expression in a suitable microorganism is a prerequisite and it is also an important choice between microbial host and expression systems.

Microorganisms synthesize numerous enzymes, each one having a specific function.Intracellular enzymes operate inside the cell in a protected and highly structured environment,while extracellular enzymes are secreted from the cell, thus working in the medium surrounding the microorganism. Here in our study the microbe selected for biotransformation is producing extracellular enzyme and responsible for splitting of oil into fatty acid and glycerol.

2.4.3.3 Cloning of genes:

Once an enzyme has been found and its amino acid sequence or the nucleic acid sequence of an enzyme protein, analysed, it can use surgical precision of genetic engineering or cloning of genes. The enzyme can be engineered; genetic variants can be constructed and selected based on new desired properties. The enzymes from the different classes vary greatly in their requirements. Today however the combination of different hosts (bacteria, yeast, and filamentous fungi) expression systems and plasmids theoretically allows hundreds of different possibilities to express and produce an enzyme [Schmidt 2004, Nevalainen 2005]. This variety of possibilities of cloning of genes and enzyme production potentially allows solutions to be developed. Here in our study we have isolated the gene responsible for lipase activity and cloned into *E.coli* to make its cloned product by avoiding the maintenance of anaerobic condition.

2.4.3.4 Systems Biology:

The development in bioinformatics will allow the study of complex biological systems by a combination of high-throughput experimental techniques with in silico model and experimental design. The integration of wet and in silico experimentation can be used for a new approach for strain improvement. The sequencing of the genomes and corresponding computer programs to search homologies are also a prerequisite. Systems biology is the attempt to not only integrate and analyze a variety of biochemical information but also actually predict a phenotype based on models which are fed with data from disparate data sets [Mack 2004].

Numerous research works have been carried out in the recent past for fatty acid production. Some of the references which are extensively used in this study are presented in the following Table 2.8. It represents various sources and microorganisms which produce different types of fatty acids. In this study, the production of lauric acid has been considered which comes under medium chain saturated fatty acid.

Table 2.8: Important References for fatty acid production

Sl. No	Scientist name	Source	Organism	Product	Year
1.	Hou C.T	Olive oil	*Acetobacter*	Oleic acid	2005
2.	Hosokawa.M	Soyabean oil	*E.Coli*	Linoleic acid	2005
3.	Kriger et. al.	Toluene	*Desulfo sp.*	Myristic acid	2006
4.	Chang et. al.	Triolein	*P.Aeruginosa*	octadecenoic acid	2007
3.	Kim et al.	Olive oil	*Aspergillus niger*	Palmitic acid	2008
7.	Gomes et. al.	Castor oil	*Yarrowia lipolytica*	γ-decalactone	2009
6.	Shinsuke Marumotto et.al.	Bergapten	*G.Cingulata*	Hydrocoumaric acid	2010
8.	Georgiana et. al.	Coconut fat	*Yarrowia lipolytica*	MCF	2012

Fatty acids are widely used as raw materials [Mun Rahman et. al. 2008] and monoacylglycerol is used as an emulsifying agent in the food, cosmetic and pharmaceutical industries [Snape et. al. 1996]. Recently diacylglycerol has received much attention as healthy cooking oil because it has a biological activity to prevent the accumulation of body fat and to lower the level of cholesterol in the blood [Soni et. al. 2001, Murase et al. 2002]. At present, the Colgate-Emery method has been industrially used for the hydrolysis of triglycerides [Kent et. al. 1974]. This process utilizes steam of high-temperature (523 K) and high-pressure (5.00×106 Pa), resulting in high energy consumption and thermal damage of the products. Recently a hydrolysis method using microbial lipase has been proposed instead of the Colgate-Emery method [Villeneuve et. al. 2000, Xu 2003]. The enzymatic hydrolysis is conducted under mild condition (at room temperature and atmospheric pressure). Therefore the above problems can be overcome by this method. Furthermore the microbial enzymes have substrate and positional specificities [Sharma et. al. 2001, Muralidhar et. al. 2001], so that the side reactions such as saponification, polymerization and oxidation are prevented to enhance the yield of the desired product.

CHAPTER-3

MATERIALS AND METHODS

This chapter is entirely describes the various experimental protocols followed, materials and resources utilized and a short description of logic behind idea of pursuing the said experiments wherever found essential. The chapter is segregated into four main sections for ease of explaining sequentially and logically. A comprehensive description commonly pertaining to all the following sections are elucidated below.

3.1 Chemicals:

Pure and analytical grade chemicals used in all experiments including media preparation for microbial culture growth and culture maintenance. Nutrients used for microbial culture and the testing kits were supplied by M/s Hi Media chemicals, India. Virgin Coconut oil and olive oil was collected from sector-II market of Rourkela, Odisha, India. Other chemicals and nutrient salts procured from M/s Merck Chemical and Sigma Aldrich, India. The molecular biology and genetic engineering products were supplied by Thermo scientific, India.

3.2 Glassware and Instruments:

All glassware's (Conical flasks, Measuring cylinders, Beakers, Petriplates and Test tubes etc.) purchased from Borosil, Rankem and Loba life. The instruments and apparatus used throughout the experiment listed in Table 3.1

.

Table 3.1: List of instruments used in the present work

Instrument	Make	Function	Operation conditions or specification
Analytical balance	Sartorius (BS223S)	Weight measurement	1mg - 100g
p^H meter	Systronics (361)	Measurement of pH	p^H 1 to 12
Vertical autoclave	Test master	Sterilization of nutrient media	121°C temperature, 15 psi pressure, 15 min.
Laminar airflow chamber	Zhichen (ZhJH-1109C)	Aseptic Environment	• Air filter: HEPA • Filter efficiency: FS209E, Class 100. • Air Velocity: 0.3-0.6m/s • Visible Light source: Fluorescent Lamp • UV light source: 8watt, mercury lamp (254nm)

BOD incubator	Vikram scientific instruments	Incubation of cultures	Temperature range: 5 - 70 ºC.
Incubator shaker	Environmental orbital Shaker	Shaking of culture flasks used in degradation study	• Speed range: 50 - 200 rpm. • Temperature: 5 - 70 ºC.
Spectrophotometer (UV/Vis)	Jasco (V-530)	Estimation of biomass and analysis of organic compounds	• Bacterial Biomass: 600 nm • TPH analysis: 228 nm • Anthrone reagent: 620 nm • Biuret reagent: 540 nm
Fermenter	IIC Industrial corporation	Biotransformation (Lauric acid Prod^{n})	2 Litre capacity, Automatic pH, Temp controlled.
Scanning Electron Microscope	JEOL (JSM-6480 LV)	To study the clear morphology about the structure and shape of the microbial cells with dimensions.	• Magnification: up to 500k. • Resolution : 3 nm • Detector: Everhardt Thornley secondary electron detector and Solid state backscattered detector. • X-Ray Analysis: Oxford Instruments ISIS 310 system with "windowless" detector. • Light element analysis: silicon detector with ATW.
Optical microscope	Hund (H-600)	Examining the morphology of microbial cells	100x, 250x, 400x and 1000x magnification.
Fourier Transform Infrared Spectroscopy (FTIR)	Perkin Elmer (3500)	Analyze the organic functional groups present in the Biosurfactant sample	• Measured range: wave number 600- 4000 cm^{-1}. • Resolution: 4 cm^{-1}
Micro Centrifuge	Remi (RM12C)	Separation of biomass from the culture broth	8000 RPM for 10 min
Vortex shaker (Spinix)	Tarson (3020)	Mixing of solution in test tube or micro centrifuge tube	• Ambient temperature of 4°C to 65°C • Position at least 30 cm from walls
Uv-Transillumintor	Genei (T20)	visualize the sample under UV light	• Peaks with ultraviolet (300-365 nm) • uniformity is <5%
Dissolve oxygen(DO) meter	Hach (HQ 10)	Checking for dissolve oxygen in solution	• Temperature (32 to 122°F, 0 to 50°C) • Measuring Range: 0.01 to 20.00 mg/L (ppm); 0 to 200%

			• saturation • Response Time at 20° C: To 95% of measured value, <30 seconds. • Measuring Principle: Luminescence decay rate
Digital colony counter	EI Products (D12)	Measure the number of microbial colonies	• Dimension: L 274 x B 320 x 167 mm • Magnification :110 mm
Gas Chromatography (GC)	Shimadzu (QP 2010)	Analysis of Hydrocarbon present in oil	• Column : VF-5ms • Length : 30.0m • Diameter : 0.25mm • Film Thickness : 0.25um • Oven Temperature : 70˚C • Injector Temperature: 240˚C • Injection Mode : Split • Split Ratio :10 • Flow control mode : Linear velocity • Column Flow : 1.51ml/min • Carrier gas : Helium • Gas Purity: 99.9995% purity
Horizontal Gel electrophoresis	Bio-Rad (SubCell® 96)	Separation and analyzing of Nucleic acid(DNA)	• Electrophoresis conditions: 50 V, 37 mA (initial). • Gel: Agarose, 4 mm thick. • Run time: 2-3hours • Buffer: 50 mM Tris acetate, 5 mM ETBr (pH 8.0)
PCR (Thermal Cycler)	Applied Biosystems (Veriti™)	Amplifying DNA samples so as to form enough copies	• 0.2 mL alloy • 96-well Fast, 0.1 ml • Max block ramp rate: up to 5.0 °C/sec • Temperature Uniformity: <0.5^0C (20 sec after reaching 95^0C) • Networking Software: VeritiLink™ Remote management software
Gel documentation system	Bio-Rad (Gel Doc™ XR)	Imaging and documentation of nucleic acid	• Illumination control: 3 modes (trans-UV, trans white, epi-white) • Image resolution: 4 Megapixels • Dynamic range: >3.0 orders of magnitude • Pixel density(gray leve):4096

DNA sequence analyzer(Genetic analyzer)	Applied Biosystems (3730 xl)	Analysis of DNA sequence and fragment	• Active temperature control between 18˚C to 70˚C • Humidity: 20 to 80% • Sample Volumes for 384-Well Sample Plates: 5-30 µL • Argon-ion multi-line, single mode laser: primary excitation lines 488 and 514.5 nm. • 98.5% base calling accuracy, less than 2% N's, using pGEM-3Zf(+) as template.

3.3 Experimental Methodology:

3.3.1 Sampling for microbial isolation:

Microorganisms used in the biotransformation study were isolated from three sites, namely sand and water samples from oil spilled site of Gokharkuda beach, Ganjam district Odisha. Soil samples from the local oil shop, Sector II, Rourkela Odisha; soil samples from the garden soil, NIT, Rourkela, Odisha, India.

3.3.2 Enrichment of Microbial community:

Soil samples of garden soil and local oil shop were inoculated in a jar containing 500ml Minimal salts media (MSM) (g 1^{-1}): 64g of Na_2HPO_4, 2.5g of NaCl, 5.0g of NH_4Cl, 15g of KH_2PO_4, 2ml of1M $MgSO_4$ and 100 µl of 1M $Cacl_2$[web ref. 9]. 5 ml of olive oill was used as a carbon source and left alone for two months under room conditions [Adekunle and Adebambo, 2007] .

3.3.3 Isolation of pure microbial cultures:

Microbial isolation experiments was performed in solid Minimal salts media (MSM) containing 1.5% agar and 1% of the virgin coconut oil as a sole carbon source. The medium was autoclaved before inoculation. Rushikulya beach sand samples and broth samples from microbial enriched jar was serially diluted and 1ml aliquot of 10^{-4} dilution samples was added to sterile petri plate by spread plate technique. Agar plates were incubated for four days at room temperature (28 - 37ºC) at a pH of 7.4 ± 0.2 .

3.3.4 Carbon source utilization efficiency:

Isolated pure cultures were tested for their ability to grow on solid MSM with 1% of the following carbon source: coconut and olive oil. Agar plates were incubated for one to two weeks at room temperature (28 - 37ºC) at a pH of 7.4 ± 0.2.

3.3.5 Sub culturing of pure cultures and inoculum preparation:

Isolated pure cultures were sub-cultured at an interval of every 15 days and the inoculum used in the biotransformation study was acclimatized using coconut oil as a carbon source.

3.3.6 Characterization of microorganism:

The different stains isolated from three different sampling sites were characterized,A bacterial starain that utilized olive oil and coconut oil as its carbon source showed good results. It had an ability to convert the substrate into desired product. Microbial identification procedure was carried out for that strain. Isolated microbial strain was characterized to identify the *genus* & *species*, family it belongs. Characterization based on Biochemical Test (morphological, physiological and biochemical) and 16s rDNA based molecular technique was followed.

3.3.6.1 Biochemical characterization:

The Biochemical test (morphological, physiological and biochemical) results were compared with Bergey's manual of determinative bacteriology. The Biochemical test for microbial species was carried out at Microbial Type Culture Collection and Gene Bank (MTCC)**,** Chandigarh**,** Punjab, India.

3.3.6.2 16s rDNA molecular characterization:

The 16s rDNA based molecular technique was performed at Xcelris genomics, Ahmedabad, Gujurat, India. The detailed experimental procedure for 16s rDNA based molecular characterization can be found in Appendix-N and step wise experimental procedure for 16s rDNA molecular technique [Rodicio et. al. 2004] is described below:

- DNA was isolated from the culture using DNA Purification Kit (Qiagen).
- Its quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA was observed.

- The 16S rDNA gene fragment was amplified by PCR from genomic DNA using 16S rDNA gene universal primers: 8F and 1492R [Sacchi et. al. 2002, Maniatis et. al. 1989], 8F: (5' AGA GTT TGA TCC TGG CTC AG 3'), 1492R: (5' ACG GCT ACC TTG TTA CGA CTT 3') and a single discrete PCR amplicon band was observed when resolved on agarose gel.
- The PCR amplicon was purified using Qiagen Mini elute Gel extraction kit according to the manufactures protocol to remove contaminants.
- The concentration of the purified DNA was determined and was subjected to automated DNA sequencing on ABI 3730xl Genetic Analyzer (Applied Biosystems, USA) and Sequencing was carried out using BigDye® Terminator v3.1 Cycle sequencing kit, following manufacturer's protocol.
- Electrophoresis and data analysis was carried out on the ABI 3730xl Genetic Analyzer using appropriate Module Basecaller, Dyeset/Primer and Matrix files.
- Consensus sequence of bacteria 'GB-1' rDNA gene was generated from forward and reverse sequence data using aligner software.
- The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genebank database. Based on maximum identity score, first ten sequences were selected and aligned using multiple alignment software program Clustal W and Distance matrix was generated using RDP database.
- The evolutionary history and phylogenetic analysis tree were done by using MEGA-4 software [[Tamura K et al., 2007].

3.3.7 Dissolved oxygen estimation:

Two types of process condition were maintained for the biotransformation study i.e. aerobic and anaerobic condition. Anaerobic condition was maintained by purging nitrogen gas into the broth. So, it is necessity to check the dissolve oxygen level present in the solution after purging of nitrogen gas to confirm the process condition. The Dissolved oxygen (DO) content of the broth was measured by a dissolved oxygen meter (Hach-HQ10) [NPDES 18th Edition of Standard Methods for the Examination of Water and Wastewater]. DO meter was equipped with a DO sensor which was immersed in the broth to measure the DO content. In another way the presence of dissolve oxygen was detected by methylene blue. Methylene blue colour changes from blue to colour less when dissolve oxygen present in the broth was zero after purging nitrogen gas.

3.3.8 Bacterial growth curve estimation:

Bacterial growth curves were tested for the both aerobic and anaerobic condition. The bacterial strains were grown in MSM broth at pH-7, Temperature (37^0C) in three successive 24-h subcultures immediately prior to the experiments. 5% of the inoculum was added to the broth. 2% of substrate was added to the main solution. Nitrogen gas was purged into the chamber to maintain anaerobic condition. The same process parameters were used for aerobic and anaerobic condition. The samples were collected in every 8 hours interval until the death phase. The collected samples were centrifuged at 8000 RPM for 10 mins. The supernatant was discarded and same amount of distilled water was added to it. The turbidity of the cultures was measured at 600 nm by the spectrophotometer. The graph of Time vs Optical density (OD) gave the growth curve of bacteria [Dalgaard paw et. al. 1994].

3.3.9 Estimation of microbial biomass:

The quantitative determination of bacterial populations is most widely established by two methods namely standard or viable method. Spectrophotometric (turbidimetric) analysis is the standard method and Plate count method is the viable cell count method. Although these methods observed same yield, yet distinct differences are visible. The standard plate count method is an indirect measurement of cell density and reveals information related only to live bacteria. The spectrophotometric analysis is based on turbidity and indirectly measures all bacteria (cell biomass), dead and alive.

***3.3.9.1 Standard biomass analysis*:**

Cultured broth (1ml) was centrifuged at 8000 RPM for 10 min and the obtained cell pellet was washed with distilled water and is centrifuged. Finally after centrifugation cell pellet was suspended in 1ml distilled water and bacterial biomass concentration was estimated by comparing the optical density (OD) observance at 600nm wavelength with the standard graph plotted with the OD values of different cells concentrations [Experimental process Biotechnology protocols, 2007]. Using the dry weight of the biomass obtained after centrifugation, different concentration of biomasss was obtained.

3.3.9.2 Viable cell count method:

Bacterial solution of known dilution and quantity solution was inoculated on the nutrient agar plate using spread plate technique. The plates were incubated at 32^0C for 24 hours in BOD incubator. At the end of the incubation period, the cells on the petri plates were counted to estimate the number of cell per milliliter using digital colony counter. The numbers of colonies were multiplied with the dilution factor and amount of microbial solution was platted over the nutrient agar [Web Ref 11].

3.3.10 Biotransformation study:

The biotransformation study was carried out in shaker flask for both aerobic and anaerobic process condition with suitable substrate (Coconut oil) to achieve our target product (Lauric acid). The reaction was carried out for eight days(from growth curve) using isolated bacterial strain in MSM broth at pH-7.4± 0.2, Temperature (37^0C), 2% substrate and 5% inoculum for both the process. At the end of the day the product was recovered with the help of n-hexane and further processed and confirmed through by FTIR and GC-MS analysis.

3.3.10.1 FTIR Analysis:

The organic functional groups present in the product of both the process were determined using FTIR analysis. The analysis was carried out using FTIR-3500 spectrophotometer. To measure the absorption spectra, the product containing solution was dropped on the sodium chloride crystal (Nacl) or Potassium bromide crystal(Kbr) at a resolution of 4 cm^{-1} and measurement wave length range from 400 to 4000 cm^{-1} [Saher M, 2011]. The FTIR analysis was carried out for the primary conformation of our desired product (Lauric acid).

3.3.10.2 GC-MS Analysis:

The GC-MS analysis was carried out for confirm our product both quantitatively cum qualitatively. The product was recovered from both aerobic and anaerobic process, through a solvent and it was subjected to GC-MS analysis for detection and conformation [Alessia et. al. 2006]. The conditions maintained to operate the GC-MS analysis the following:

<u>GC CONDITION</u>

Column Oven Temperature	:	70˚C
Injector Temperature	:	240˚C
Injection Mode	:	Split
Split Ratio	:	10
Flow Control Mode	:	Linear Velocity
Column Flow	:	1.51 ml/min

Carrier Gas : Helium 99.9995% purity

Injection volume : 1 microlitre

Column Oven Temperature Program

Rate	Temperature (˚C)	Hold Time (min)
-	70.0	2.0
10	300	7.0 [32.0 mts total]

COLUMN

VF-5ms

Length : 30.0m

Diameter : 0.25mm

Film thickness : 0.25µm

MS CONDITION

Ion source temp : 200 °C

Interface temp : 240°C

Scan range : 40 – 1000 m/z

Solvent cut time : 5mins

MS start time : 5(min)

MS end time : 32 (min)

Ionization : EI (-70ev)

Scan speed : 2000

MS LIBRARY : NIST08s, WILEY8, FAME

3.3.11 Optimization Study (Lauric acid):

Literature review depicts the influence of various factors on biotransformation process such as temperature, p^H, Substrate concentration, agitation (RPM) etc. Our primary study suggest that anaerobic condition is more efficient than aerobic condition for production of lauric acid by the isolated strain. Moreover these parameters also greatly influence in the production of lauric acid. These parameters were optimized so as to obtain maximum growth of the microorganisms and lauric acid production in anaerobic condition. The growth was analyzed in terms of biomass. The process parameters which influence the most in the production of lauric acid (biotransformation) were optimized by Gray based Taguchi method using

Minitab-16 software. The factors (Process parameters) and their levels (different range) were given in Table 3.2.

Table 3.2: Factors and their levels used in the lauric acid production optimization study

Factor	Optimization parameters	Unit	Level 1	Level 2	Level 3	Level 4	Level 5
A	Temperature	^{o}C	25	30	35	40	45
B	pH	-	5	6	7	8	9
C	Substrate Concentration	%	1.0	2.0	3.0	4.0	5.0
D	Agitation	RPM	50	75	100	125	150

The grey relational analysis based on the grey system theory was used to solve the complicated interrelationships among the multiple responses effectively. In a grey system data pre-processing is the primary stage since the range and the unit in one data sequence may differ from the other. Data pre-processing is a means of transferring the original sequence to a comparable sequence. Depending on the characteristics of a data sequence, there are various methodologies of data pre-processing available for this analysis [Tosun et. al. 2010]

The optimization of the process was performed in the following steps:

1. Normalizing the experimental results of fungal biomass and residual oil content for all experimental run.
2. Calculating the Grey Relational Coefficient (GRC).
3. Calculating the Grey Relational Grade (GRG) by averaging the GRC's.
4. Performing statistical analysis of variance (ANOVA) for the input parameters with the GRG and identifying the parameters that significantly affected the process.
5. Selecting the optimal levels of process parameters.

The indication of the better performances of microbial strain in biotransformation study was biomass yield "higher the better". In the analysis of Grey relation for "higher the better" response normalization used *Equation 1*

Response normalization for larger is better condition. $x_i^{*}(k)$

$$x_i^*(k) = \frac{x_i(k) - \min x_i(k)}{\max x_i(k) - \min x_i(k)} \tag{1}$$

Where $x_i^*(k)$ and $x_i(k)$ were the normalized data and observed data, respectively, from i^{th} experiment using k^{th} response. The smallest and the largest value of $x_i(k)$ for the k^{th} response were min $x_i(k)$ and max $x_i(k)$, respectively. After pre-processing the data, the Grey relation coefficient $\xi_i(k)$ for the k^{th} response characteristics in the i^{th} experiment was expressed as follows.

$$\xi_i(k) = \frac{\Delta \min + \xi \, \Delta \max}{\Delta_i(k) + \xi \, \Delta\max} \tag{2}$$

Where $\Delta i(k)$ was the k^{th} value in Δ_i different data series. Δ_{max} and Δ_{min} were the global maximum and minimum values in the different series, respectively. The distinguishing coefficient was selected by decision maker's judgment, and different distinguishing coefficients usually provided different GRG results. The most preferred value of ξ was considered as 0.5. After calculating GRC, for n number of responses, the GRG (γ) was being calculated using Equation 3. ξ_i

$$\gamma = \frac{1}{n} \sum_{i=1}^{n} \xi_i(k) \tag{3}$$

The magnitude of γ reflects the overall degree of standardized deviation of the i^{th} original data series from the reference data series. In general, a scale item with a high value of γ indicates that the respondents, as a whole, have a higher degree of favorable consensus on the particular item.

3.3.12 Lauric acid Production in a bench top fermenter:

A fermenter provides a controlled, contained and homogenous environment in which fermentation of bacterial cultures can be performed safely and practically to achieve particular objectives. Laboratory scale production of the lauric acid was carried out in a 2litre capacity(1 ltr as working volume) bench-top fermenter (IIC, Labeast Bio fermenter) maintaining the optimized parameter values (By Taguchi method) at Temperature- 40^0C, pH-8, Substrate concentration- 2%, Agitation-150 rpm in anaerobic condition for 192 hours.

The fermenter was equipped with pH sensor, internal cooling and heating system, magnetic agitator, DO sensor, dosing bottles, nitrogen gas inlet port, sampling port etc. The fermenter vessel and all other components were autoclaved prior to the assembling and fitting. Freshly grown culture of 'GB-1' was used as inoculum total 5% of fermentation broth (MSM). Temperature and pH maintenance is very important in the production process, where a little change in these parameters will affect the physiochemical characteristics of the cells resulting in the shift in the metabolic pathway. Temperature detection and adjustment was done by thermocouple and sensor connected to it by circulating hot and cold water inside the coil. pH detection and adjustment was done by using pH probe and dosing acid or alkali whenever there is a shift in the pH using a fixed speed peristaltic pump. 1N HCl and 1N NaOH were used for the purpose. Foaming was observed in the broth and controlled by adding antifoam agent (Antifoam Y30 emulsion, Sigma Aldrich) was used in minimum amount (0.01%).

Sampling was performed in daily basis for 8 days and headed for estimation of biomass and lauric acid to estimate the percentage of lauric acid produced per day. The biomass was estimated spectrophotometrically and lauric acid percentage was estimated through GC-MS analysis. Substrate kinetics (For both aerobic and anaerobic condition) and Product kinetics (for anaerobic condition) were calculated. The Biotransformation efficiency calculation was given in Appendix-M.

3.3.13 Enzyme detection:

The isolated strain, which was responsible for lauric acid production was biochemically and genetically characterized and confirmed as *Pseudomonas aeruginosa*. From the literature review it is found that Lipases catalyzes ester synthesis and transesterification among different acyl glycerols, alcohols, esters, glycosides and amines or even among different chemical groups of the same compound [Ferrer et. al.2000, Jaeger et. al. 2002]. Bacterial extracellular lipases isolated from *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas cepacia* and *Pseudomonas glumae* are highly selective catalysts [Xie ZF 1991]. So as to confirm the enzyme produced by microbial strain the basic method of lipase detection i.e. Substrate (Tributyrin) agar plate and Rhodamine-B dye method was carried out.

3.3.13.1 Substrate agar plate method:-

Lipase positive strain was identified on Luria-Bertani-Tributyrin(LBT) agar plates. The composition of LB broth was given in Appendix-B. 1% of tributyrin was added to it. The medium was prepared by adding all constituents and then autoclaved and dispensed into sterile petridishes and allowed to solidify. After solidification well was created by punching machine (based on different diameter). Actively grown culture was centrifuged at 12,000 RPM for 10 min and 25-100µl (depending up on well diameter) culture supernatant was loaded to well. The plates were incubated at 37^0C for a period of 24-48 hours. The development of a clear zone around the well is an indication of lipolytic activity [Gupta et. al. 2003].

3.3.13.2 Rhodamine-B dye agar plate method:-

A plate assay to detect bacterial lipase in a medium containing coconut oil or triglycerol and the fluorescent dye rhodamine-B. Bacterial growth medium (MSM) was adjusted to pH 7.0, autoclaved and cooled to about 60^0C. Then 1% of coconut oil, 10 ml of rhodamine-B solution (0.001% w/v) in 90 ml growth medium was added with vigorous stirring and emulsified by mixing for 1 min with a homogenizer. 20 ml of medium was poured into each petri dish. After solidified various wells were created by punching machine (based on different diameter). Actively grown culture was centrifuged at 12,000 RPM for 10 min and 10-25µl (depending up on well diameter) culture supernatant was loaded on to different wells. The plates were incubated for 48 hours at 37^0C. Lipase enzyme detection was monitored by irradiating plates with UV light at 350nm. After 24 hours of incubation bacterial colonies began to show orange colour fluorescence, with continuing incubation time orange colour fluorescent halos were formed around the colonies [Martinez. et. al. 2001].

3.3.14 Lipase activity assay:

Lipase activity was determined for both aerobic and anaerobic process condition on dalily basis for 8 days. Lipase activity was determined by the standard p-Nitrophenol method [Karadzic et. al. 2006] with some modifications. The substrate used in this study was p-Nitrophenyl dodecanoate or laurate. The substrate solution was prepared by dissolving 0.01344gm of p-nitrophenyl dodecanoate in 50 ml of Isopropanol. The study was conducted using 1:1 ratio of substrate with double distilled water. The working standard for substrate concentration was 420µM. The enzyme solution was prepared by taking 2 ml of growth culture sample and centrifuged at 12,000 RPM for 10 min. The supernatant was collected and the biomass was discarded. The supernatant was contained the desired extracellular lipase enzyme.

The lipase assay was carried out at 37^0C in phosphate buffer (pH-7) by mixing of substrate, enzyme solution, Triton-X100, 1M Na_2CO_3 in different testubes for 10 min

Incubation time. In this study Temperature-37^0C and p^H -7 was chosen primary, since isolation of microbial strains was conducted at this Temperature and pH. The composition of enzyme, substrate and buffer can be found in Appendix-C. The amount of liberated p-Nitrophenol was measured at 410 nm and final absorbance reading was noted (Appendix-C). The amount of p-Nitrophenol released was calculated from standard graph. The standard graph for p-Nitrophenol can be found in Appendix-D. One unit of enzyme activity (IU) was defined as the amount of enzyme that liberated 1 µmol of p-Nitrophenol per minute under the standard assay conditions.

The Concentration of lipase was calculated for anaerobic condition to check the combining effect of substrate degradation, biomass growth, product formation and enzyme produced. The enzyme concentration was determined by measuring its protein concentration. The protein estimation was done by the Biuret assay. The step wise procedure of Biuret assay method of protein estimation and standard graph can be found in Appendix-E and Appendix-F.

3.3.15 Optimization of Lipase activity:

The Enzyme activity mostly depends upon the composition of the substrate and medium [Kim et. al. 1996]. Major content of lipase production is substrate source which also act as inducer for lipase production. Since microbial lipases are often inducible enzymes [Lotti et. al. 1998] whereas, suitable carbon source and physiological condition regulates the growth of producer organism and thus the fermentation process [Rathi et. al. 2002]. There are many approaches for designing an ideal fermentation process. The traditional approach to the optimization problem is the one variable at a time method. In which all variables but one are held constant and the optimum level for this variable is determined. Using this optimum, the second variable's optimum is found, etc. [Henry et. al 1997]. Here the lipase activity was optimized by the one factor at a time approach due to mixed level parameter.

Lipase activity optimization was carried out for aerobic condition of *pseudomonas sp.* due to maximum enzyme activity than compared to anaerobic condition in previous experiment. The various parameters considered for optimization of lipase activity was Temperature, pH, Enzyme concentration (in terms of volume), substrate concentration and the time of incubation. The various parameters considered for optimization study were following: Temperature- 25ºC, 30ºC, 35ºC, 40ºC, 45ºC, 50ºC, 55ºC and 60ºC. pH- 5, 6,7 ,8, 9, 10, 11 and 12(Preparation of different pH buffer can be found in Appendix-H). Enzyme concentration (in terms of volume) - 100µl, 200µl, 300µl, 400 µl, 500µl, 600µl, 700µl, 800µl, 900µl and 1000µl. Substrate concentration- 100µM, 200µM, 300 µM, 400µM, 500µM, 600µM, 700µM, 800µM, 900µM and 1000µM. The time of incubation- 5 min, 10 min, 15 min, 20 min, 25 min, 30 min. The lipase activity estimation procedure was carried out by standard p-Nitrophenol method by taking substrate as p-nitrophenyl dodecanoate. The lipase activity estimation assay and p-Nitrophenol standard graph can be found in Appendix-C and Appendix-D.

3.3.16 An Approach for isolation of the gene responsible for lipase Production:

Isolation of lipase gene consists of different steps. They are as following:

3.3.16.1 Extraction of Genomic DNA from pure Culture **[**Experimental Techniques in Bacterial Genetics, Jones and Bartlet, 1990]:

- The bacterial sample was spun till desired amount of pellet was obtained (1ml of culture corresponding to 1 O.D).
- It was washed twice with distill water.
- The pellet was resuspended in 567µl of TE buffer [Appendix-G] by repeated pippetting and vortexing.
- In this bacterial suspension 30µl of 10% SDS and 15µl of RNase (10 mg/ml) and Proteinase K (20 mg/ml) were incubated at 37^0C for 30 minutes.
- 80µl CTAB-NaCl [Appendix-G] solution was added to the above mixture and was mixed thoroughly. It was incubated for 10 minutes at 65ºC.
- The addition of RNase (30 µg of RNase /ml) was also added initially to remove RNA Contamination.

Phenol Chloroform Treatment:

- About 250µl of Tris saturated phenol [Appendix-G] and 250µl chloroform was added to the tube after incubation.
- It was thoroughly mixed by inverting the tube carefully.
- The tube was spanned at 12,000 rpm for 10 minute & the upper layer was transferred to a fresh tube.
- Equal volume of chloroform was added & was rocked for 15 minutes.
- The tube was centrifuged at 12,000rpm for 10 minutes & the supernatant was transferred to a fresh tube.

- 1/10th volume of 3M-sodium acetate of pH 5.2 & 0.7 volume of Isopropanol was added and incubated at room temp for overnight.
- After incubation it was centrifuged at 14,000rpm for 30 minutes and supernatant was discarded.
- The pellet was washed with 70% ethanol.
- The pellet was air dried completely & dissolved in 50µl double distilled water.

3.3.16.2 Analysis of Genomic DNA by spectrophotometer:

The concentration of nucleic acids was determined by measuring at 260 nm against a blank. Interference by contaminants was recognised by the calculation of a "ratio". Since proteins absorb at 280 nm, the ratio A260/A280 was used to estimate the purity of nucleic acid. DNA quantification was done using spectrophotometric measurement of UV absorption at wavelengths 260 and 280 nm. The ratio provides indication of protein contamination respectively [Manning, 1991]. Pure DNA should have a ratio of approximately 1.8 [Medberry 2004].

The DNA concentration was also calculated using the formula,

DNA concentration (µg /ml) = OD at 260 nm × dilution times × standard value

(The standard value is 50 because 1 unit OD corresponds to a concentration of 50 µg/ml whereas dilution time is the no. of times the DNA is diluted).

3.3.16.3 Analysis of Genomic DNA by Agarose gel electrophoresis:

Most DNA extracts are not reasonably pure and therefore estimates of concentration using spectrophotometric measurements of UV absorption may be misleading because of the interference by RNA or non-nucleic acids contaminants. The standard method used to separate, identify and purify DNA fragments is electrophoresis through agarose gels [Vinod 2004]. The technique is simple, rapid to perform and capable of resolving mixtures of DNA fragments. The migration rate of DNA through agarose gels is additionally dependent upon the molecular size of the DNA, In our study, quantification of the DNA was achieved by running the DNA sample on 1 % agarose gel stained with ethidium bromide (0.5 µg/ml) (Ethidium bromide is a fluorescent chemical that intercalates between base pairs in a double stranded DNA molecule). Aliquots of the DNA extracts are loaded [7 µl of DNA mix (5 µl of each DNA sample and 2 µl of loading dye)] alongside a uncut lambda DNA standards. The electrophoresis was carried out in TAE buffer (Tris acetate EDTA).

High molecular weight DNA will appear as a well-resolved band alongside the lambda DNA bands whilst the smearing below the band indicates mechanical or chemical degradation. A rough estimate of DNA content (±10 ng) was obtained by comparing band intensities of the DNA extract and the standards by eye in gel documentation system.

(A) (B)

***Figure 3.1:* (A)** *Horizontal Gel electrophoresis (BIO-RAD) &* **(B)** *Gel documentation system (BIO-RAD)*

3.3.16.4: PCR amplification of the gene responsible for Lipase production:

In this study, DNA template was amplified for detection of gene coding for lipase enzymes production. The study was carried out in presence of primers which was earlier reported for amplification of this gene. PCR was used to amplify small regions of lipase genes directly from chromosomal DNA. This was achieved by using highly degenerate consensus primers to the oxyanion hole [Jaeger et. al., 1994] and active-site regions of lipase genes to amplify fragments lipases.

PCR (Polymerase Chain Reaction) is a technique widely used in molecular biology, used to amplify specific regions of a DNA strand (the DNA target). It derives its name from one of its key components, a DNA polymerase (Taq-DNA Polymerase) used to amplify a piece of DNA by in vitro enzymatic replication. After a thorough literature study, the primer which amplifies the gene responsible for the lipase production was selected for the study. All the chemicals used in this study were obtained from Thermo scientific chemicals, India.The primer used in this study was

Forward Primer: 5’-CATATGATGAAAKGCTGYCGGGT-3’

Reverse primer: 5'-GGATCCTTAAGGCCGCAARCTCGCCA-3' [Sifour et. al. 2010].

The microbes were screened for the presence of the lipase gene using primer reported by available literature. The composition of the reaction mixture (refer Appendix-I) PCR method mainly consists of three types of Temperature profile.

Initialization step: This step consists of heating the reaction to a temperature of 94–96 °C which was held for 1–9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR [Sharkey et. al. 1994].

- *Denaturation step*: This step was the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.
- *Annealing step*: The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the melting temperature of the primers used. The annealing temperature of primer in this study was 56^0C.The calculation for annealing temperature can be found from Appendix-J. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation.
- *Extension/elongation step*: The temperature at this step depends on the DNA polymerase. Taq polymerase has its optimum activity temperature at 72–80 °C [Lawyer et. al. 1993] and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. The extension time used in our study was 7 min. As a rule-of-thumb, at its optimum temperature the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

The PCR cycle used in our study was repeated for 30 times. Different temperature profile of PCR cycle in our study is described in Figure 3.2.

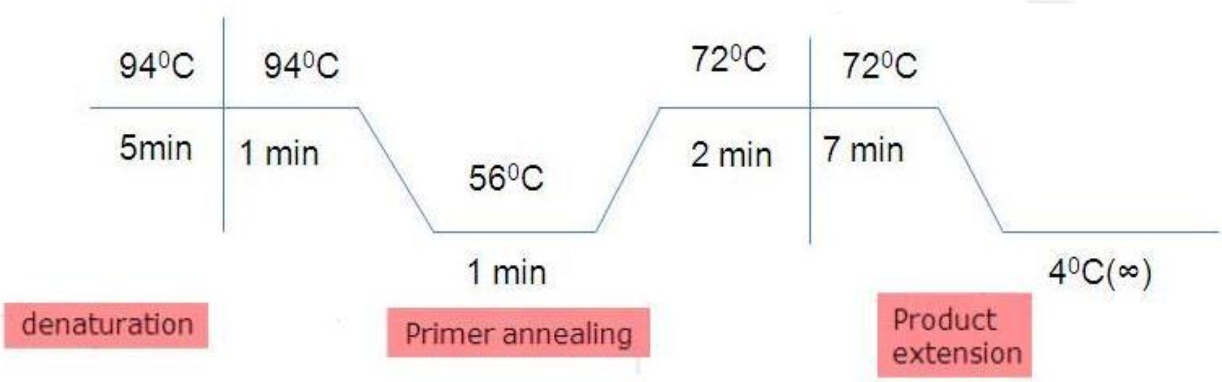


Figure 3.2: *PCR Cycle for detection of Lipase gene*

Figure 3.3: *Thermal Cycler used in this study (PCR, Applied Biosystems)*

3.3.16.5 Isolation of gene responsible for Lipase Production (Recombinant DNA Technology [Yuping et. al. 1992] :

DNA molecules constructed outside the living cells that is in vitro by joining natural or synthetic DNA segments that can replicate in a living cell is called as recomobinant DNA technology.

Goals of Recombinant DNA Technology:

a) To isolate and characterize a gene

b) To make desired alterations in one or more isolated genes

c) To return altered genes to living cells

Overview of Recombinant DNA procedure:

Isolate DNA ⟶ Cut with restriction enzymes ⟶ Ligate into cloning vector à transform recombinant DNA molecule into host cell ⟶ each transformed cell will divide many, each of which carries the recombinant DNA molecule (DNA clone). The basic tools of recombinant DNA technology are Genomic DNA, Restriction endonucleases, plasmid, DNA ligases, host vector and many more. The various methods for making recombinant DNA in our study are as follows:

3.3.16.5.1 Isolation of Genomic DNA

The *Pseudomonas sp.* genomic DNA was isolated by the standard DNA extraction method of bacteria **[**Experimental Techniques in Bacterial Genetics, Jones and Bartlet, 1990] as explained in previous topic.

3.3.16.5.2 Restriction Digestion:

DNA can be cut into large fragments by mechanical shearing but restriction enzymes are the standard scissors of molecular genetics. Restriction enzymes (RE) are endonucleases that recognize specific nucleotide sequences in the DNA and break the DNA chain at those points. Most cut at specific palindromic sites in the DNA (sequence that is the same on both antiparallel DNA strands). These cuts staggered which generate “sticky or overhanging ends” .or a blunt which generate flush ends.

*Bam*HI (from *Bacillus amyloliquefaciens*) is a type II restriction endonuclease, having the capacity for recognizing short sequences (6 b.p.) of DNA and specifically cleaving at a target site. Each *Bam*HI subunit makes the majority of its backbone contacts with the phosphates of a DNA half site but base pair contacts are made between each *Bam*HI subunit and nitrogenous bases in the major groove of the opposite DNA half site. In our study *Bam*-HI (Thermo scientific) was used for restriction digestion. The restriction digestion protocol used in our study was followed by manufacturer’s protocol. The components which were added for digestion is indicated orderly in following manner:

Table 3.3: Reaction composition for restriction digestion

Chemical	Quantity(µl)
Genomic DNA	7
Buffer	1
Enzyme	0.1
Nuclease free water	1.9
Total	10

- Samples were mixed gently and spinned down briefly.
- Samples were incubated at the optimal reaction temperature (37^0C) for 0hr, 3hr, 6hr and overnight incubation.
- So, four samples were incubated for four different hour containing 10µl each reaction volume.

3.3.16.5.3 Ligation:

DNA ligation is the process of joining together two DNA molecule ends (either from the same or different molecules). Specifically, it involves creating a phosphodiester bond bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another. This reaction was usually catalyzed by a DNA ligase enzyme. The enzyme, ligate the DNA fragments having blunt or overhanging, complementary, sticky ends. Ligases are vital enzymes required for physiological cellular processes such as DNA replication, repair of damaged DNA and recombination. In our study *T4 DNA ligase* (Thermo scientific) was used for ligation. It is the most commonly used DNA ligase for molecular biology techniques and can ligate both ends.

After selecting the maximum digested sample, it was subjected for ligation by following manufacturer's protocol (Thermo scientific):

Table 3.4: Reaction mixture composition for ligation

Chemical	Quantity(µl)
Ligation buffer	2
DNA Sample	5
pUC-19 digetsed with *Bam*-HI	0.5
T4 DNA- Ligase(1U)	1
Nuclease free water	11.5

Reaction mixture was prepared using different composition (Table 3.4). The reaction mixture was incubated for one hour at 22^{0}C. After incubation 5µl of ligation mixture was subjected to transformation study.

In our study pUC-19 (Thermo scientific) vector was used as plasmid cloning vectors. pUC-19 is one of the most widely used vector molecules as the recombinants. pUC19 is a commonly used plasmid cloning vector in *E.coli*. The molecule is a small double-stranded circle, 2686 base pairs in length and has a high copy number. It has one *amp*^{R} gene (ampicillin resistance gene) and an N-terminal fragment of β-galactosidase (*lac Z*) gene of *E.coii*. The multiple cloning site (MCS) region is split into the *lac Z* gene (codons 6–7 of *lac Z* are replaced by MCS), where various restriction sites for many restriction endonucleases are present. pUC vector is small but has a high copy number. The high copy number of pUC plasmids is a result of the lack of the *rop* gene and a single point mutation in rep of pMB1. The *lac Z* gene codes for β-galactosidase. The recognition sites for HindIII, SphI, PstI, SalI, XbaI, BamHI, SmaI, KpnI, SacI and EcoRI restriction enzymes were derived from the vector M13mp19.

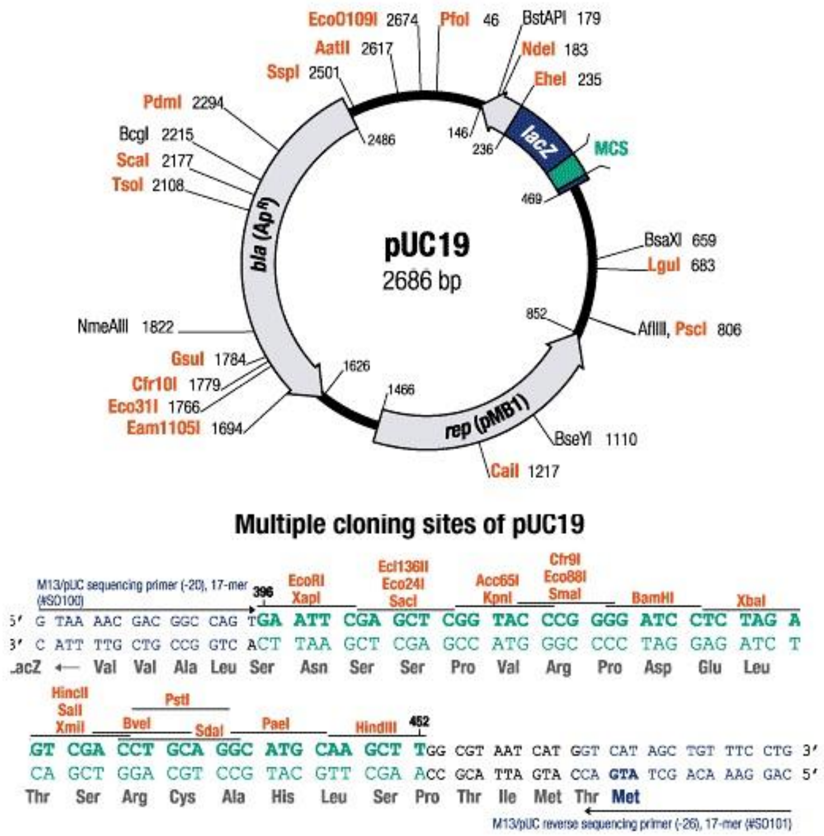


Figure 3.4: *Structure of pUC-19 plasmid vectror (Thermo scientific)*

3.3.16.5.4 Transformation:

A vector is any DNA that can take foreign DNA by ligation using complementary site in the plasmid. These sites are generated by digesting the DNA & vector with the same restriction enzymes. Vectors contain genetic markers that confer strong growth advantages under selective conditions. The plasmid DNA containing foreign DNA as insert is usually introduced artificially by transformation to a competent cell. Selection is then carried out by screening for the colonies those have acquired the plasmid and hence grow on antibiotic containing plates. Routinely used antibiotics include ampicillin, kanamycin, chloramphenicol, carbenicillin & tetracycline.

In our study the transformation was carried out using Thermo scientific transform aid bacterial transformation kit. It is one of the new method for rapid preparation of chemically competent *E.Coli* cells from overnight bacterial culture. Manufacturer's transformation protocol was followed in our study and is described below:

- The day before the transformation seed overnight culture was prepared by inoculating 2ml of C-medium with a single bacterial colony(not older than 10 days). And incubated the culture overnight at 37^0C in a shaker.
- Pre warm LB agar (2%) plates were supplemented with appropriate antibiotic in an incubator with 37^0C.
- T Solution preparation: T-Solution (A)-250µl and T-Solution (B) - 250µl was mixed thoroughly in a separate tube and kept on ice.
- 150µl of the overnight bacterial culture was added to 1.5ml of pre-warmed C-Medium and incubated for 20 min at 37^0C in a shaker.
- After incubation bacterial cells were pelleted by 1 min centrifugation and the supernatant was discarded.
- Pelleted bacterial cells were resuspended in 300µl of T-solution and incubated on ice for 5 min.
- T-solution containing bacterial cells were centrifuged again for 1min and supernatant was discarded.
- Cell pellets were resuspended in 120µl of T-solution and incubated on ice for 5min.
- 5µl of ligation mixture was added (containing 10-100 ng vector DNA) and 1µl of supercoiled DNA (10-100 pg) was added into new micro centrifuge tubes and chilled on ice for 2 min.

- 50µl of the prepared cells were added to tube containing DNA and mixed it. Then incubated on ice for 5 min.
- The mixture solution was plated immediately on prewarmed LB-antibiotic agar plates and incubated overnight at 37^0C.
- Control(100 pg of supercoiled (pUC-19) was also taken for transformation study. The transformation efficiency was calculated by formula given in Appendix-K.
- The detailed receipe for Ampicillin, X-gal, IPTG and LB antibiotic plates can be found in Appendix-K.

3.3.16.5.5 Construction of Genomic DNA Library:

A genomic is a collection of DNA from a single organism, ideally though not necessarily containing its entire genomic DNA sequence. The DNA from the source organism of interest is divided into multiple fragments and packaged within cloning vectors such that each carries a portion of it. Genomic library also called as clone bank or gene bank.

In our study, the genomic library from *Pseudomonas aeruginosa* was prepared using pUC-19 as cloning vector and *E.coli* as host. *E. coli* transformations were carried out according to manufacturer's protocol (Thermo scientific). Genomic DNA of *Pseudomonas aeruginosa* was restricted with *Bam*HI and ligated into pUC-19 plasmid linearized with *Bam*HI [Qurrat-ul-Ain et.al. 2003]. The genomic DNA fragments so ligated was transformed into *E. coli by* bacterial aid transformation kit (Thermo scientific) and plated on agar-ampicillin plates.

3.3.17 Antimicrobial activity of lauric acid:

Antimicrobial (AM) agent are class of compounds which capable to kill microorganisms or suppresses their multiplication or growth. There are numerous AM agents that exist and widely used in a variety of applications in the food, pharmaceutical and cosmetic industries. Lauric acid is potential to use as the antimicrobial agent because it sources are from local sources, coconut.

The liquid culture test was used to determine the antimicrobial activity of the test compounds by optical density which provides information on microbial growth curve, thus being more sensitive than the agar diffusion method [Han 2003, Mann et. al. 1998]. The liquid culture growth was measured spectrophotometrically by taking absorbance at 600nm. The antimicrobial activity of Lauric acid were tested with two bacterial strain *E.Coli* (Gram-ve) and *Bacillus Subtilis* (Gram +ve). Various amount of lauric acid volume were added ranging from 0.2, 0.4, 0.6, 0.8 & 1ml were dissolved in 5%DMSO. The sample was drawn in every 2 hours difference for 24 hour to check the microbial biomass growth. 1ml of bacterial suspension was taken as positive control and 1 ml of 5% DMSO [Georgiana et. al. 2012] was taken as negative control. The microbial growth curve was plotted between Time vs. optical density [Salleh et. al. 2009] to determine the growth curve pattern for checking of antimicrobial activity of lauric acid.

CHAPTER-4

RESULTS AND DISCUSSION

This Chapter details about the various findings we came across while carrying out various experimental procedure for our study and findings are authenticated by various characterization methods.

4.1 Isolation and Characterization of Microorganism

4.1.1 Sample collection from natural oil contaminated sites:

Most of the microbes species present in the environment genetically have an efficiency to utilize the natural oil or hydrocarbons as a carbon source. This ability comes to expression only when they are grown in that specific environment. Sample collection from petroleum oil's contaminated sites will facilitate to isolate microbial strains with hydrocarbon degradation [Li et al., 2000] (Length, 2010) and natural oil utilizing efficiency[Darshan et al. 2011**]**. So, samples have been collected from three sites, namely sand and water samples from oil spilled site of Gokharkuda beach, Ganjam district Odisha. Soil samples from the local oil shop, Sector II, Rourkela Odisha; soil samples from the garden soil, NIT, Rourkela, Odisha, India.

4.1.2. Isolation of Microorganisms:

To augment and isolate the efficient natural oil utilizing microorganisms soil sample collected from the garden soil was inoculated (5%) in 500 ml Minimal salts media (MSM)[web ref. 9] containing 1% olive oil as sole carbon source and left for 2 months at room temperature. At the end of 2 months some microbial colony growth was observed over the water sample. 500µl of 10^{-4} serial diluted microbial enriched water sample from the garden soil inoculated liquid MSM, sand and water samples collected from oil spilled site of Gokharkuda beach and soil samples from local oil shop were inoculated on solid MSM of p^H 7.4 ± 0.2 containing 1% Virgin coconut oil as a carbon source. Culture plates were incubated for 7 days at temperature 28 to 37ºC. After seven days of incubation microbial colonies were observed on the seven petri dishes (Table 4.1).

Microbial colonies grown on oil were further tested to examine the growth on higher fractions. MSM with olive and coconut oil was used as a carbon source and the culture plates were incubated at 28 to 37ºC temperature for 7 days and 15 days respectively. After incubation period growth of microbial colonies were observed on the petri dishes (Table 4.1).

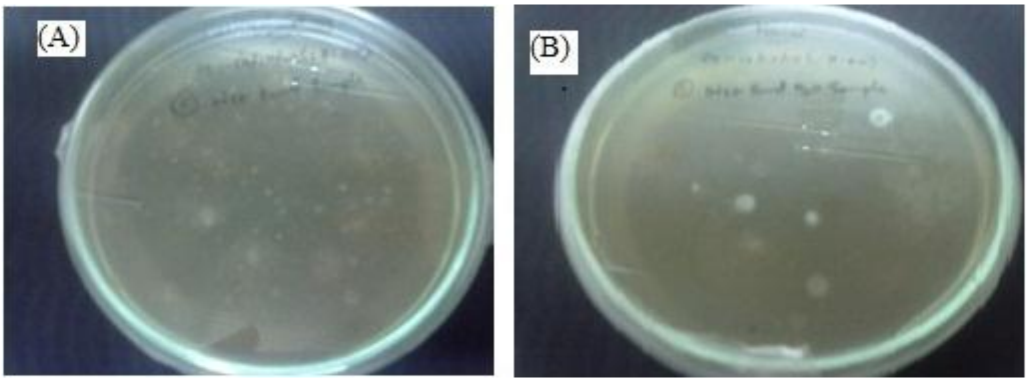


Figure 4.1: *(A) Microbial growth from Gokharkuda beach sample (Olive oil)*

.... (B) Microbial growth from Gokharkuda beach sample (Coconut oil)

Table 4.1: Microbial colonies isolated from various sampling sites

Sample Name	Sampling site	Microorganism Type	Growth(No of colonies)	
			Olive oil	Coconut oil
LOS-1	Local oil shop	Bacteria	25	8
GS-1	Garden Soil	Bacteria	18	2
LOS-2	Local oil shop	Bacteria	9	2
GB-1	Gokharkuda beach	Bacteria	39	14
GB-2	Gokharkuda beach	Bacteria	20	1
GB-3	Gokharkuda beach	Bacteria	17	1
GB-4	River water	Bacteria	14	

4.1.3 Characterization of Microorganisms:

4.1.3.1 Bacterial Characterization (Biochemical method):

Among the different stains isolated from three different sampling sites, one bacterial strain have shown good results in utilizing olive and coconut oil as a carbon source and converting substrate into our desired product. Microbial identification procedures havebeen carried out for one strain. According to the Bergey's manual of systematic bacteriology bacterial strains are characterized on the basis of morphological features (Table.4.2), standard physiological tests (Table.4.3) and biochemical tests (Table.4.4) [Bayoumi, 2009] [Oberhofer et al., 1977] and characterization followed by using 16S rDNA based molecular technique.

In the morphological characterization study, Grams staining '*GB-1*' bacterial cells retained pink safranin colour and they are gram negative and rod shaped. It is due to the thin peptidoglycan layer in the periplasm does not retain the dark stain, and the pink safranin counter stain stains the peptidoglycan layer in gram-negative bacteria. *GB-1* are rod shaped Bacillus cells and bacterial colony produces a green colour water soluble pigment called Pyocyanin.'*GB-1*' bacterial cells posses locomotion due to the presence of flagella. *GB-1* bacterial cells exhibit growth between 25°C to 42°C temperatures. So, they can be considered under class Mesophiles. *GB-1* bacterial cells exhibit very good growth rate in p^H range from 7.0 to 9.0 and still it exhibited growth up to an higher p^H 12.0. So they can be considered under class obligate alkalophiles. '*GB-1*' bacterial cells exhibit growth up to 12% NaCl concentration. So, they are considered as Halopiles (Moderate halophiles) [Web Ref 10].

Table 4.2: Morphological test results of bacterial strains

Tests	GB-1
Colony configuration	Circular
Colony margin	Wavy
Colony elevation	Flat
Colony surface	Smooth
Colony texture	slimy
Pigment production	green pigment color
Opacity	Opaque
Gram's reaction	-
Cell shape	Rod
Spore(s)	-
Motility	Motile

'+': Positive; '-': Negative

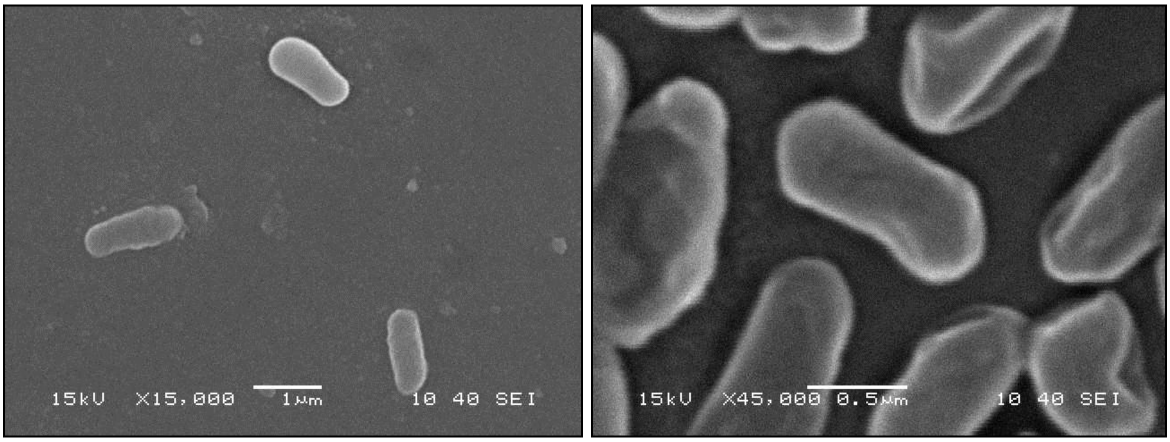


Figure 4.2: *SEM pictures of 'GB-1' bacterial cells*

Table 4.3: Physiological test results of bacterial strain

Growth at Temperatures	GB-1	Growth at pH	GB-1	Growth on NaCl (%)	GB-1
4°C	-	pH 5.0	-	2.0	+
15°C	-	pH 6.0	+	4.0	+
25°C	+	pH 7.0	+	6.0	+
30°C	+	pH 8.0	+	8.0	+
37°C	+	pH 9.0	+	10.0	+
42°C	+	pH 10.0	+	11.0	+
55°C	-	pH 11.0	+	12.0	+
		pH 12.0	+		

'+': growth observed; '-': growth not observed

Biochemical tests were conducted to identify the bacterial strain (Table.4.4). The tests results are discussed below.

- Methyl-red test is performed to evaluate the mixed acid fermentation by '*GB-1*' bacterial cells. In this test bacterial cells were grown on Methyl red, Voges-Proskauer (MR-VP) broth containing glucose, peptone, and a phosphate buffer. Organisms that perform mixed-acid fermentation produce enough acid to overcome the buffering capacity of the broth, so a decrease in p^H results. After incubation, the p^H indicator Methyl Red is added to the broth that shows yellow color which indicates p^H above 6.0. So it is considered as negative result.

- Voges-Proskauer test is done to determine whether an organism can produce acetylmethylcarbinol (acetoin) from fermentation of glucose. The cultures are incubated in Clark and Lubb's medium which contain alpha-naphtol (5%) and potassium hydroxide (40%). After incubation it doesn't produce any pink reddish colour which indicates negative result.
- Casein hydrolysis test is done to examine the production of exoenzyme *Casesase* which hydrolyses the Casein, a complex protein present in milk agar. '*GB-1*' inoculated plates didn't produce any clear zones which indicate the negative result.
- The citrate test performed to detects the ability of a microorganism to use citrate as the sole source of carbon. '*GB-1*' Bacteria are inoculated on a medium containing sodium citrate and a p^H indicator bromothymol blue is used. The culture tubes produced blue colour which indicated the production of enzyme *citritase*. Enzyme *citritase* breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down to pyruvate and carbon dioxide (CO_2). Production of sodium bicarbonate ($NaHCO_3$) as well as ammonia (NH_3) from the use of sodium citrate and ammonium salts results in alkaline p^H. This positive result indicated by change in the color of the medium from green to blue.
- In nitrate reductases test, broth of the '*GB-1*' strain remain colour less which indicates that bacterial cells are able to reduce nitrate (NO_3^-) to nitrite (NO_2^-) using anaerobic respiration and was supported with positive result.
- The indole test is performed on bacterial species '*GB-1*' to determine the ability of the organism to split indole from the amino acid tryptophan. Yellow color in the surface alcohol layer of the bacterial strain inoculated broths represents the result as negative. It may be due to lack of production of intracellular enzymes *tryptophanase* that split indole from the amino acid tryptophan.
- Arginine test is performed to distinguish the *Pseudomonas* species from other microbial species. *Pseudomonas* species produces the enzyme, arginine dihydrolase. It releases ammonium from arginine which results in alkalinity p^H indicated by p^H indicator phenol red, that turns a dark pink color under oil as contrast to the orange pink of arginine dihydrolase-negative cultures. Here '*GB-1*' strain exhibited positive results which indicated that bacterial strain belongs to *Pseudomonas* species.
- Gelatin test is done to tests the ability of an organism to produce an exoenzyme, called *gelatinase* that hydrolyzes gelatin present in the media. '*GB-1*' strains inoculated tube indicates liquefies the solid gelatin present in the media. The positive result indicates

production of gelatinase enzyme to hydrolyse gelatin into smaller polypeptides, peptides, and amino acids that can cross the cell membrane and be utilized by the organism.

- Starch hydrolysis is performed to tests the ability of '*GB-1*' strain to produce certain exoenzymes, including *α-amylase* or *oligo-1,6-glucosidase*, that hydrolyzes starch. A clear zone around the bacterial colony observed indicates that the organism has hydrolyzed starch by producing *α-amylase* or *oligo-1,6-glucosidase* and bacterial strain was supported by negative result.
- Catalase test is performed to check the ability of '*GB-1*' strain to produce enzyme *catalase* that breaks hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). When the bacterial strain were inoculated bubbling was seen which is due to the evolution of O_2 gas. It indicates that both the bacterial strains produce enzyme *catalase* with positive test result.
- In oxidase test broth of the '*GB-1*' strain colour changed to blue which indicates that bacterial cells produce the enzyme *cytochrome oxidase*. *Cytochrome oxidase* participates in the electron transport chain by transfer of electrons from a donor molecule to oxygen. The colour change is due to the oxidation of a reducing agent, chromogenic present in oxidase reagent. So the test result for both the strains was positive.
- On McConkey agar the '*GB-1*' strain has grown by utilizing the peptone instead of lactose content of agar. So called non lactose fermentation (NLF) which was indicated by the formation of random circular colonies without change in colour to pink.
- Along with the biochemical tests some fermentation tests are also done to identify the acid production characteristics of the microorganism on various carbon sources which are discussed in Table 4.4.
- On observing the results of morphological tests, physiological tests and biochemical tests bacterial strain (*GB-1*) was considered as ***Pseudomonas Sp.***

Table 4.4: Biochemical test results of bacterial strains

Test	G	Acid Production from	G
Methyl red test	-	Dulcitol	-
Voges Proskauer test	-	Trehalose	-
Casein hydrolysis	-	Sorbitol	-
Citrate	+	Raffinose	-
Nitrate	+	Melibiose	+
Indole	-	Salicin	-

Arganine	+	Sucrose	+
Gelatin hydrolysis	+	Rhamnose	-
Starch hydrolysis	-	Fructose	+
Esculin hydrolysis	-	Cellobiose	-
Catalase test	+	Galactose	+
Oxidase test	+	Inositol	-
Growth on McConkey	NLF		

'+': growth observed; '-': growth not observed, 'NLF': Non-Lactose Fermentation

4.1.3.2 Bacterial Characterization (16S rDNA based Technique):

4.1.3.2.1 Experimental Result:

- DNA was isolated from the bacterial culture. Its quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA was observed.
- Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose Gel (Gel Image).
- The PCR amplicon was purified to remove contaminants.
- Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

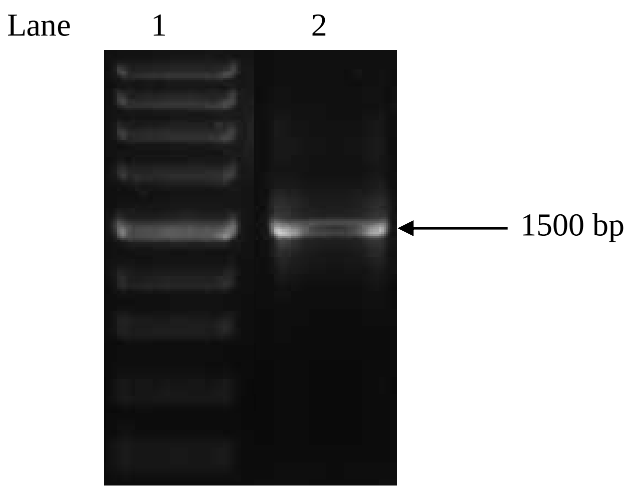


***Figure 4.3**-Gel Image of 16SrDNA*

Lane 1: DNA marker, Lane 2: 16S rDNA amplicon band

- Consensus sequence of **1334bp** 16S rDNA gene was generated from forward and reverse sequence data using aligner software.

- The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W.

Table 4.5: Sequence Producing Significant Alignments

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HQ844440.1	*Pseudomonas aeruginosa* strain JSYM27	2459	2459	100%	0.0	99%
JQ900528.1	*Pseudomonas aeruginosa* strain N141	2420	2420	100%	0.0	99%
JQ900520.1	*Pseudomonas aeruginosa* strain N86	2420	2420	100%	0.0	99%
JQ900511.1	*Pseudomonas aeruginosa* strain N72	2420	2420	100%	0.0	99%
JX149546.1	*Bacterium* P2A	2420	2420	100%	0.0	99%
JX149543.1	*Bacterium* H1C	2420	2420	100%	0.0	99%
JX122841.1	*Pseudomonas sp*. J-36	2420	2420	100%	0.0	99%
HE978271.1	*Pseudomonas aeruginosa* , strain DSM 50071T	2420	2420	100%	0.0	99%
JX093570.1	*Pseudomonas aeruginosa* strain XM2	2420	2420	100%	0.0	99%
JX093569.1	*Pseudomonas aeruginosa* strain XM1	2420	2420	100%	0.0	99%

4.1.3.2.2 Distance Matrix:

Distance matrices was used in phylogeny as non-parametric distance methods were originally applied to phenetic data using a matrix of pairwise distances. These distances are then reconciled to produce a tree (a phylogram, with informative branch lengths). The Ribosomal Database Project (RDP) provides data, tools and services related to ribosomal RNA sequences. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4.

Table 4.6: Distance matrix using RDP database

Sample	1		0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
HQ844440.1	2	0.001		0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
JQ900528.1	3	0.006	0.005		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
JQ900520.1	4	0.006	0.005	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000
JQ900511.1	5	0.006	0.005	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000
JX149546.1	6	0.006	0.005	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000
JX149543.1	7	0.006	0.005	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000
JX122841.1	8	0.006	0.005	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000
HE97871.1	9	0.006	0.005	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000
JX093570.1	10	0.006	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000
JX093569.1	11	0.006	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	

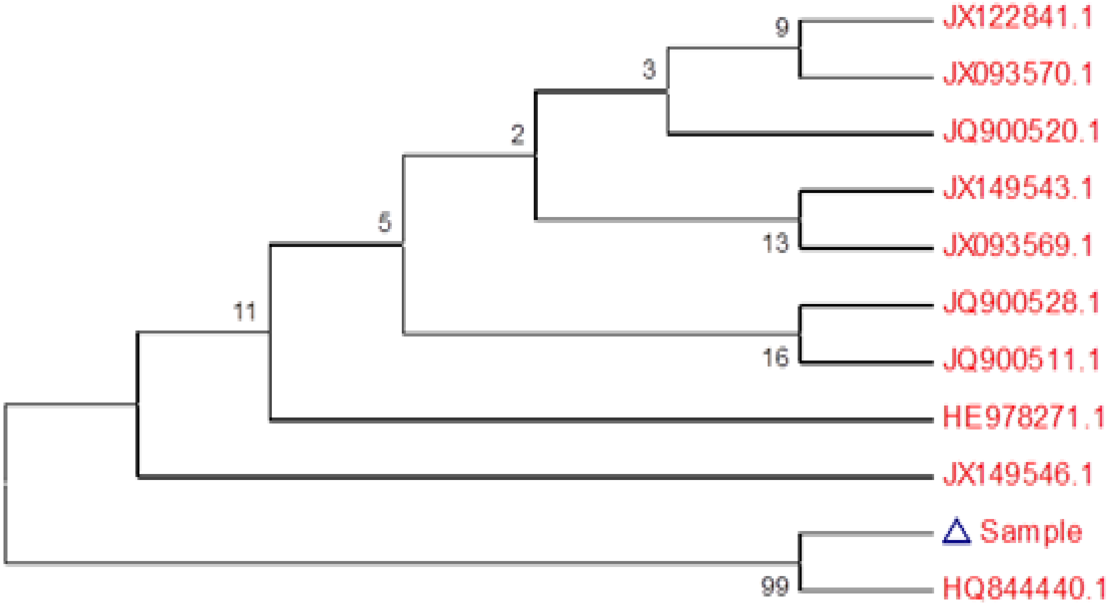


Figure 4.4: *Phylogenetic Tree (Evolutionary relationship)*

The culture, which was labeled as Sample was found to be *Pseudomonas aeruginosa* based on nucleotide homology and phylogenetic analysis. Information about other close homologs for the microbe can be found from the above alignment view table 4.5. Our consensus sequence has registered in NCBI sequence submission database with GeneBank Accession Number: KC310862. The strain was named and registered as *VBP01*.

The evolutionary history was inferred using the Neighbor-Joining method [Saitou N et al., 1987]. The bootstrap consensus tree inferred from 500 replicates [Felsenstein J, 1985] was considered to represent the evolutionary history of the taxa analyzed [Felsenstein J, 1985]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches [Felsenstein J, 1985]. The evolutionary distances were computed using the Kimura 2-parameter method [Kimura M., 1980] and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1334 positions in the final dataset. Phylogenetic analyses were conducted using MEGA4 [Tamura K et al., 2007].

4.2 Bacterial growth curve estimation:

Bacterial growth curve was studied for aerobic and anaerobic condition in a shaker flask culture. Anaerobic condition was maintained and checked by DO meter as explained in chapter no-3. Bacterial growth curve shows that the graphical representation of the life-cycle of a bacterial cell (and its population) over a given period of time. Binary fission and other cell division processes bring about an increase in the number of cells in a population. Population growth was represented through the growth curve of the biomass. Microorganisms are cultivated in liquid medium, are incubated in a closed culture vessel with a single batch of medium. In absence of fresh medium during incubation, nutrient concentrations decline and concentrations of wastes increase. Graphical representation of the microbial growth is observed from the plot of time versus biomass optical density.

The resulting curve depicts four distinct phases. They were mainly lag, log or exponential, stationary and death phase at different experimental condition [Figure 4.5(aerobic) and 4.6(anaerobic)]

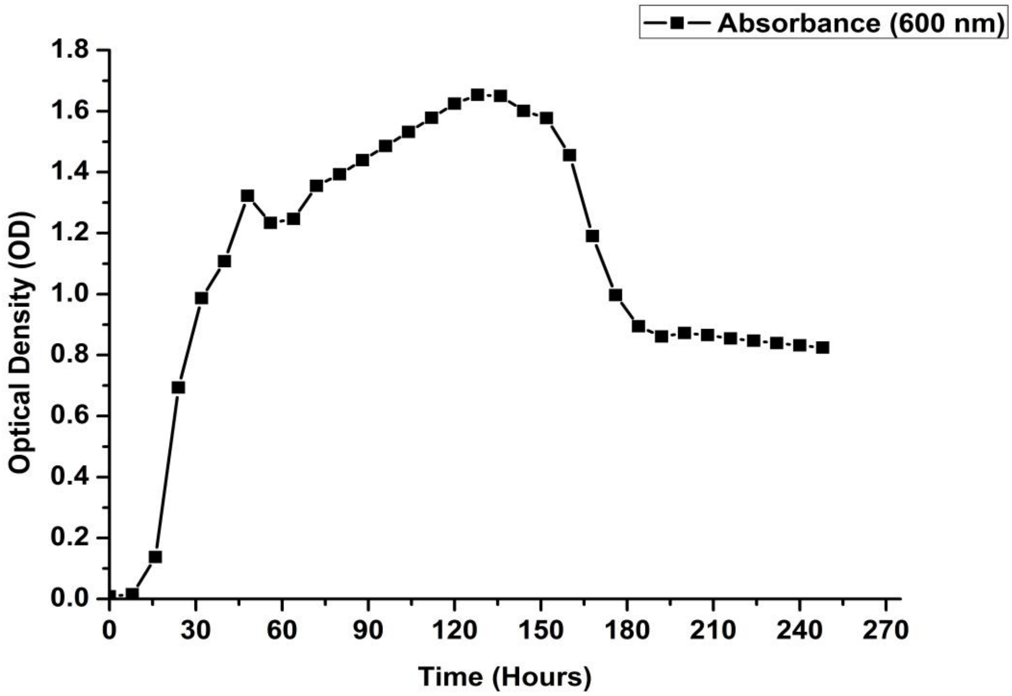


Figure 4.5: *Growth Curve of Pseudomonas aeruginosa (Aerobic)*

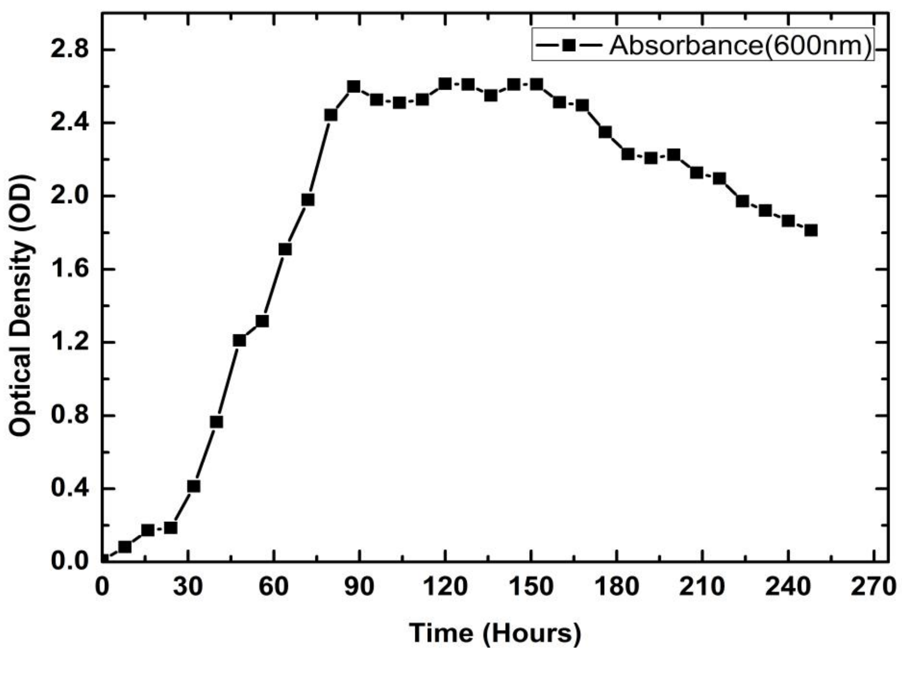


Figure 4.6: *Growth Curve of Pseudomonas aeruginosa (Anaerobic)*

In the lag phase, the organisms were adjusting to their environment. The proteins (enzymes) necessary for energy production and binary fission was produced and the ribosomes necessary for their production was also synthesized. In case of aerobic condition the lag phase time was less than compared to anaerobic condition. It is due to its optimal metabolism condition. It may be due to in anaerobic condition as the adaptation time was more the common metabolism condition for *Pseudomonas aeruginosa* is aerobic and it takes time for adaptation of microbial population in the anaerobic condition when differs from the common metabolism process. It also depends upon the type of carbon source available. In anaerobic condition the common pathway also changed. So, the time needed for adapting changed pathway may also attribute towards increase in its adaptation time [web ref. 13].

During the exponential (log) phase microorganisms grow and divide at the maximal possible rate. Each microbe at their genetic potential depends upon the nature of the medium, and the environmental conditions. Their rate of growth was exponential during this phase i.e. they were completing the cell cycle and doubling in number at regular intervals (figure 4.5 & 4.6). The population was also most uniform in terms of chemical and physiological properties during this phase. Biochemical and physiological studies were performed using the culture at its experimental growth phase. Exponential (logarithmic) growth was the phase of growth where the growth rate is proportional to the increasing number or size in an exponential (rather than arithmetical) or logarithmic progression. The growth pattern of exponential phase in aerobic condition differs from anaerobic condition. It may be due to the variation in availability of optimum metabolism condition, cell adaptation and available of nutrients for growth [Zwietering et. al. 1990]. The total time for exponential phase in anaerobic condition was more due to availability of nutrients according to their cell growth ratio where as in aerobic condition the growth rate was high as compared to the available nutrient ratio. So, the exponential phase showing more fluctuation as compared to anaerobic condition. From the growth curve it is also observed that as soon as the completion of adaptation process the exponential growth phase is continuing rapidly until nutrients available in the system.

In the stationary phase new organisms were produce, at the same rate at which they were dying. This represents a sort of equilibrium between cell birth and cell death. The availability of nutrients and oxygen are the limiting factor and the cells were scramble to survive by making adjustments within the cell [Zwietering et. al. 1990]. In aerobic condition the time period for stationary phase is less than anaerobic condition due to more accumulation of toxic products. It directly enters into to its death phase. In case of anaerobic condition the stationary phase is longer than aerobic condition and moreover it's fluctuating due to non-availability of nutrients and cells were trying to complete their metabolic cycle with minimum condition. Moreover due to the accumulation of more toxic wastes. In death phase the no. of cells were dying due to the nutrient limitation condition. In aerobic condition the death phase occurs more rapidly than compared to anaerobic condition due to shrinkage of the cell drastically for 140 to 180 hours and then became constant.

4.3 Biotransformation study:

4.3.1 Biotransformation Study (for lauric acid production):

The initial biotransformation study was carried out for 100ml in shaker flask for both aerobic and anaerobic process condition (figure 4.7). Virgin coconut oil was taken as substrate to produce lauric acid as found from literature review. In initial study the process parameters were same for both the process i.e. Temperature-37^0C, pH-7.4± 0.2, Substrate concentration-2% and inoculum-5%. The process parameter like Temperature and pH were taken as 37^0C and 7.4 due to the isolation of microbes and primary confirmation of substrate utilization was studied in this level.

Figure 4.7: *Shaker flask experimental setup for biotransformation study (Lauric acid production) in (a) Aerobic (b) Anaerobic Condition*

The biotransformation process was carried out for 192 hour (8days) for aerobic and anaerobic condition as it is observed in microbial growth curve. The product was confirmed by using FTIR analysis and GC-MS analysis.

4.3.1.2 FTIR Analysis:

The primary confirmation of our product (lauric acid) was done using FTIR analysis by confirming the organic functional group present. FTIR analysis was done for before and after the reaction in both aerobic and anaerobic condition. The result obtained were interpreted and compared to the literature.[Fourier Transform Infrared Spectrometry by Peter R. Griffiths**,** ISBN-10 / ASIN: 0471194042].

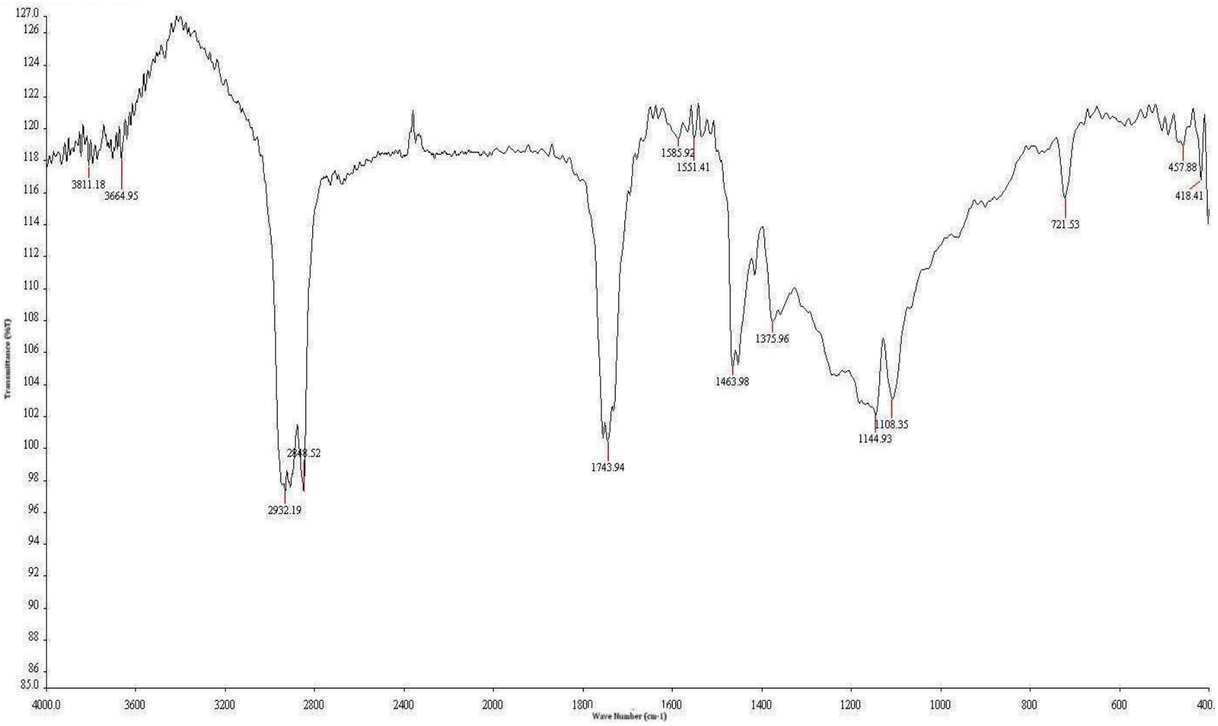


Figure 4.8: *FTIR analysis of virgin coconut oil (before biotransformation)*

From the figure 4.8 during FTIR interpretation we found that the band region from 2980-2850, 1755-1730 and 1470-1450 shows the possibility of aliphatic carboxylic acid esters. Free fatty acid was not there in the oil due to the absence of hydroxyl terminal free carboxylic acid. The band region 1750-1731 cm^{-1} shows the presence of carbonyl group (C=O).

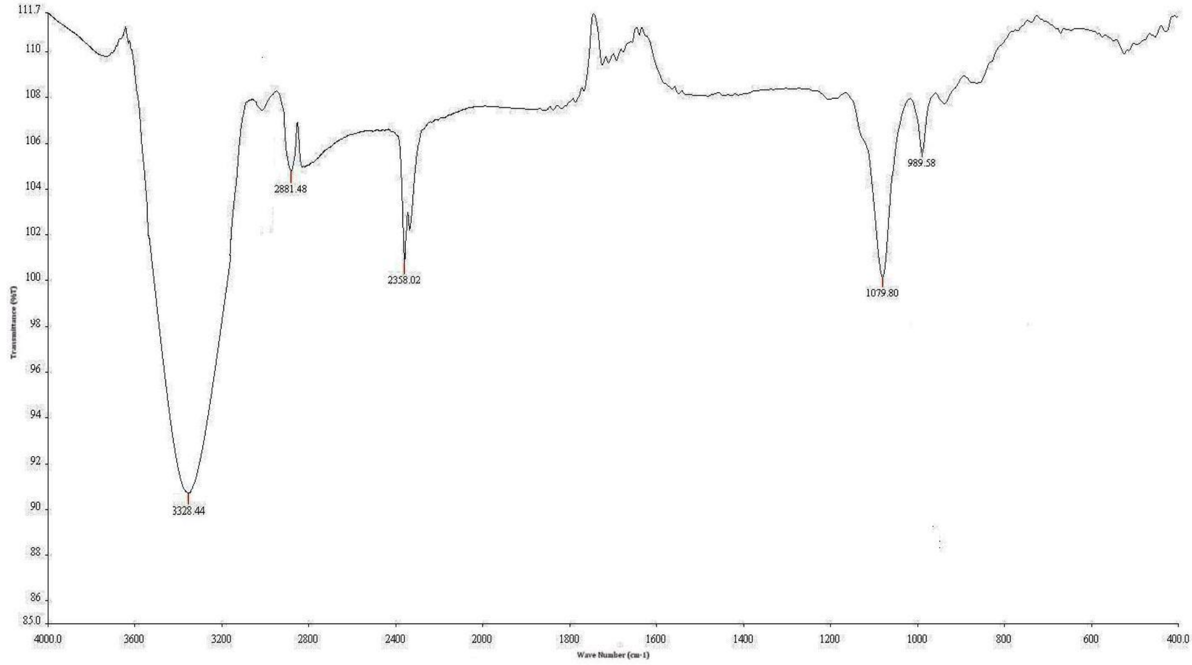


Figure 4.9: *FTIR analysis of solution after biotransformation (aerobic condition)*

From the figure 4.9 during FTIR interpretation we found that the band region 3550-3200(broad, strong) shows the presence of alcoholic group due to the OH stretching. The band region 3000-2500(broad, variable) contains the peak 2881.48. This shows that the presence of carboxylic acid OH stretching and presence of free carboxylic acid. The band region 1780-1710 also show the stretching of C=O. Hence it shows that the probable presence of free fatty acid in the sample.

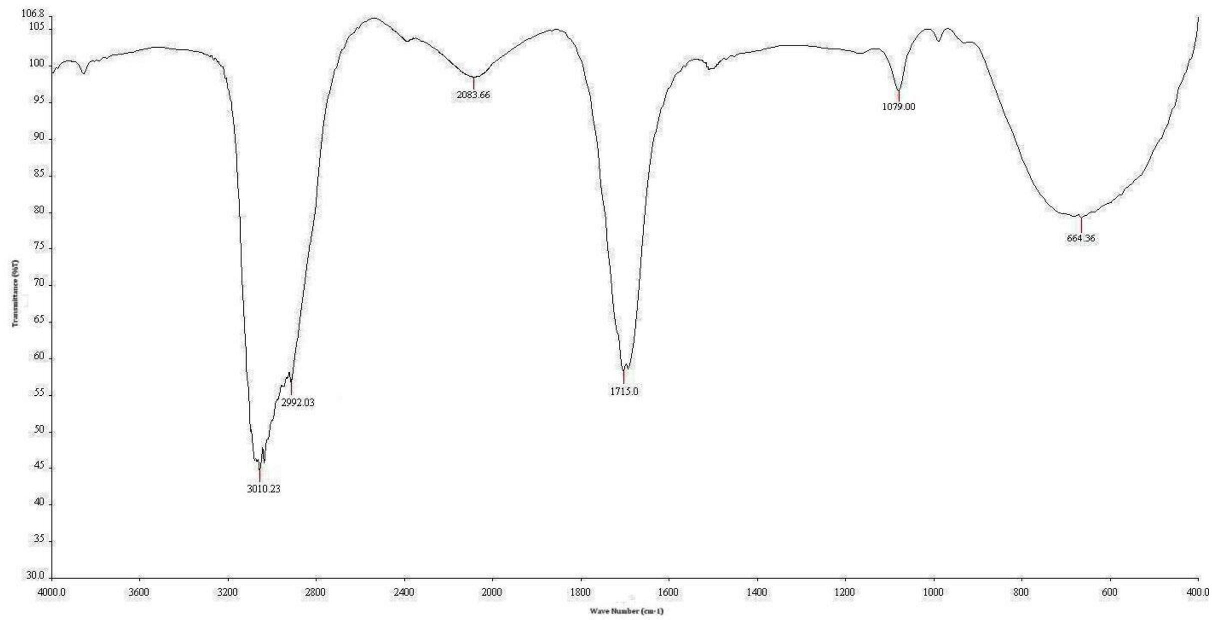


Figure 4.10: *FTIR analysis of solution after biotransformation (anaerobic condition)*

From the figure 4.10 during FTIR interpretation we found that the band region 3000-2500(broad, variable) and 1780-1710(strong) shows the stretching of hydroxyl terminal of carboxylic acid group and carbonyl stretching of carboxylic acid group respectively. The band region between 3020-3550 shows the absence of alcoholic group. Hence from our interpretation it can was confirmed that there may be the absence of glycerol or any kind of alcohol but the presence of an acid.

Primary confirmation of desired product was achieved through FTIR analysis. The presence of the major and exact compound before and after biotransformation was studied using GC-MS analysis and which is explained in next paragraph.

4.3.1.3 GC-MS Analysis:

The analysis of fatty acid by gas state radio-chromatography [James et. al. 1961] became an important part in biotransformation studies on fatty acids [Stymne et. al. 1978, Gurr 1971]. Lauric acid profile of transformed or hydrolysed coconut oil at aerobic and anaerobic condition at different time interval is presented in figure 4.12 and 4.13. The operating condition for GC-MS like type of column and its temperature, oven temperature, injection temperature etc. were already explained in materials and methods.

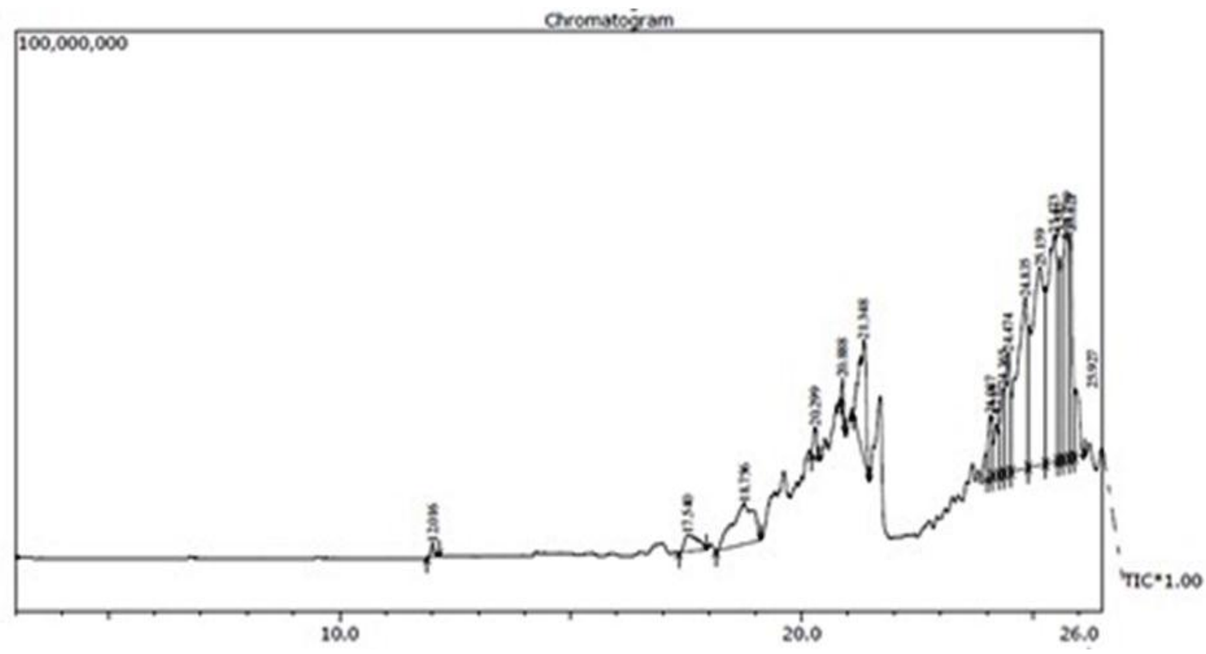


Figure 4.11: *GC analysis of Virgin coconut oil (before biotransformation)*

The above graph shows various peaks for different compound present in virgin coconut oil which was used as a substrate in our study. From MS analysis the compounds were identified. The percentage of each compound was also calculated from peak area (Appendix-L) .compounds present in sample was found from MS analysis. The major compound present in virgin coconut oil was Glycerol trilaurate (90.79%) whereas glycerol trimystrate a minor compound was present in the rest.

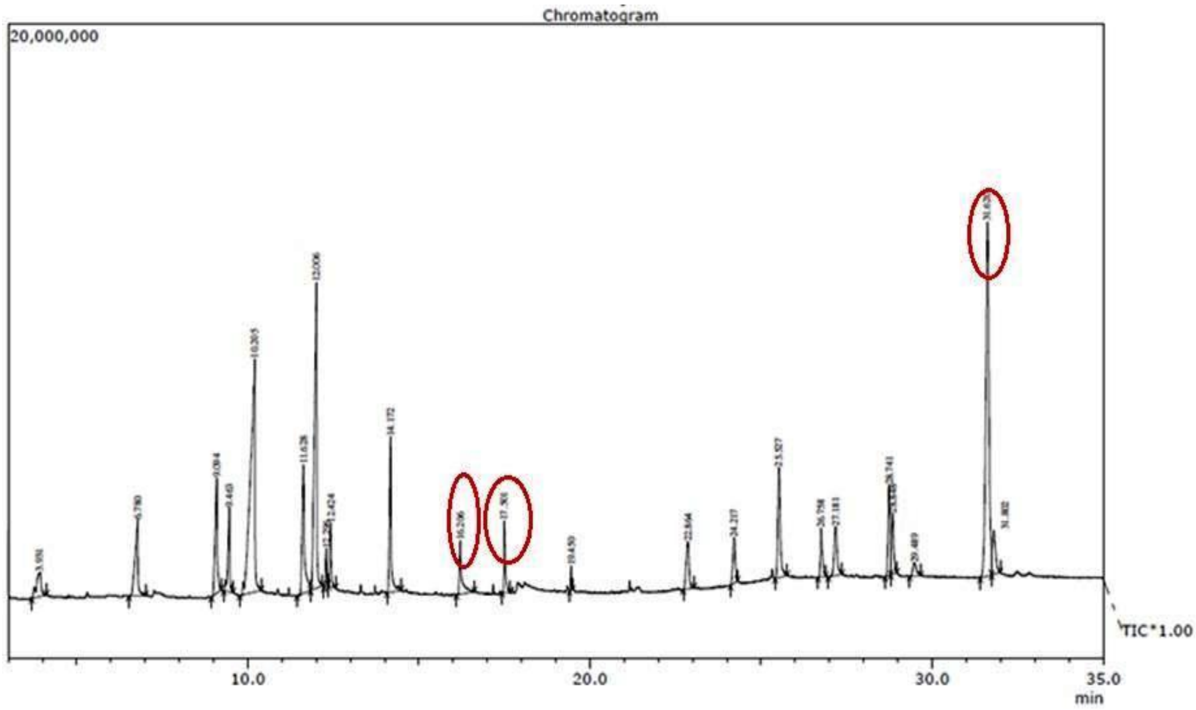


Figure 4.12: *GC analysis after reaction (aerobic condition)*

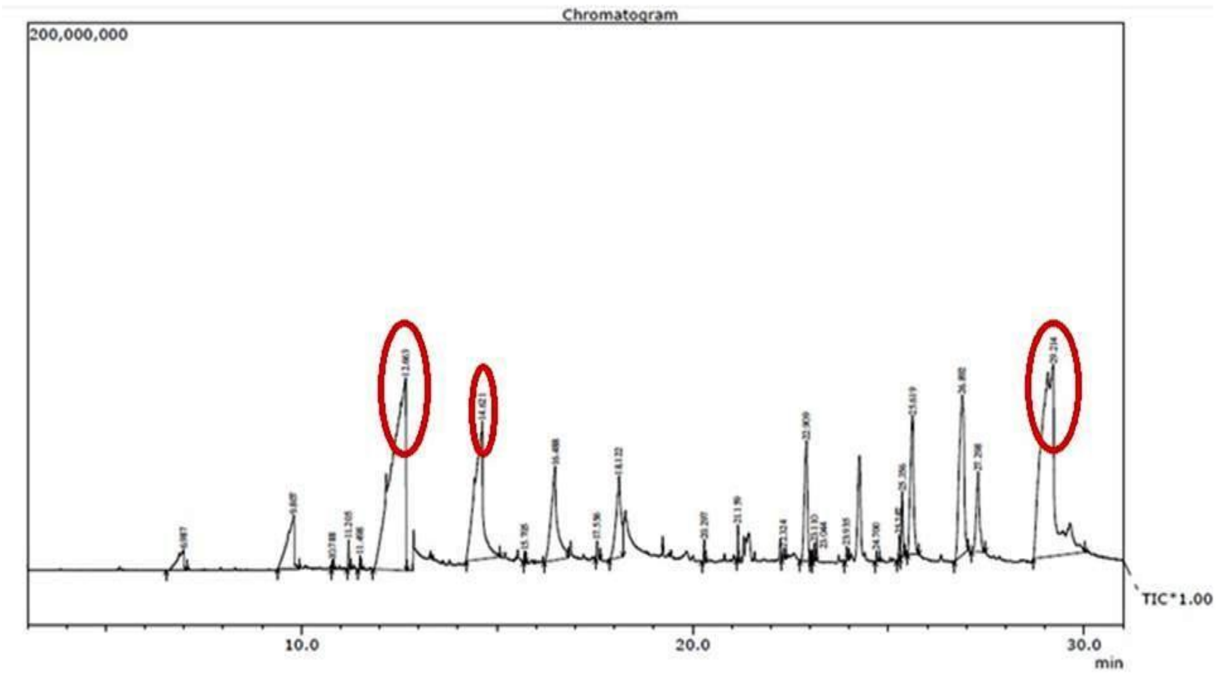


Figure 4.13: *GC analysis after reaction (anaerobic condition)*

The extraction of lipid from aqueous solution (broth) was using *n*-hexane as the developing solvent was experimented. Hexane part was separated by using separation flask which contained desired fatty acid and was subjected to GC-MS analysis. It was found that MCSFA (Medium chain saturated fatty acid) were present in higher concentration in anaerobic condition as compared to aerobic condition (From GC figure 4.12 & 4.13). The compounds were identified through MS analysis. In aerobic condition the major compounds were present such as glycerol (40.31%), glycerol trilaurate (19.49%) and lauric acid (14.09%) where as in anaerobic condition the major compound present were Lauric acid (61.98%), glycerol trilaurate (7.68%), glycerol (0.46%), minor impurities and solvent present in rest of the analysis part. In aerobic condition *Pseudomonas aeruginosa* carried out its metabolic activity in presence of oxygen. Due to its favourable condition, the process of metabolism could be accomplished easily with little consumption of oil used as carbon source. The lauric acid produced from coconut oil due to microbial lipase enzyme cleaves the glycerol and fatty acid. Presence of less lauric acid in the solution was due to fatty acid consumption by microbes to complete its regular metabolism. So, in aerobic condition glycerol quantity was more as compared to lauric acid. Presence of glycerol trilaurate in the indicates that some unconverted oil was still present in the solution.

In anaerobic condition the process of degradation of oil was little bit complex than the aerobic condition. It is established that *Pseudomonas aeruginosa* is an aerobic microbe. In anaerobic condition the growth was bit late (time consuming) due to its non-usual condition of metabolism and time required for adaptation was significant. In anaerobic condition the percentage of lauric acid produced was more whereas glycerol was in trace amount. It was attributed to the fact that *Pseudomonas aeruginosa* was forced to break the oil, seeking oxygen to survive and continue its metabolism with limited amount of lipase produced. So after getting the oxygen molecule it consumes the glycerol as carbon source and leaves free fatty acids in the solution.

In GC graph the target product of our study was rounded. It confirms that the lauric acid production was high in anaerobic condition than aerobic condition (Figure 4.12 & 4.13). So, anaerobic condition was considered as suitable condition for production of lauric acid in terms of quality and quantity in our study. The process parameter for lauric acid production was optimized to achieve maximum production in anaerobic condition.

4.4 Optimization of process parameters (Biotransformation):

Biotransformation is found to be comparatively slow than chemical transformation because it is influenced by a number of factors which include the microbial community which produce the fatty acids, temperature and nutrient availability [Ekpo et. al. 2008]. In the present study, process parameters such as Temperature, pH, substrate concentration and agitation (RPM) on the growth of microorganisms was investigated. These parameters play an important role in the growth of microorganisms, in production of fatty acid (lauric acid), intracellular and extracellular activities and in uptake of carbon source. The optimization experiments were carried out using grey based taguchi method which is explained below.

4.4.1 Grey based Taguchi approach in Optimization of lauric acid production:

The conventional one factor at a time approach of optimization is not only incapable in its interactions but often time consuming. The method becomes impractical when a large number of nutrients in the culture medium have to be considered since too many combinations have to be considered to optimize the growth medium composition. Taguchi established that statistically planned experiments are important for a successful design of parameter. Statistically designed experiments were used earlier in industry. However, Taguchi's contribution adds a new aspect to this experimentation by providing a quick and accurate way of determining optimization [Rao et. al. 2008]. Taguchi established that statistically planned experiments are essential for a successful parameter design [Swan et. al. 1998].

The statistically planned experiments (orthogonal matrix method) was performed to investigate the relationships between variables of process parameters (Temperature, pH, substrate concentration and agitation) and optimize their concentrations for microbial cells growth and production percentage of lauric acid. The orthogonal matrix method, as a result of the suitable design of factors can give effective responses and reduce performance variation [Kim. et al., 2005].

4.4.1.1 Design of orthogonal array (L_{25}):

The control factors illustrated in Table 4.7 includes four different types of process parameters such as Temperature, pH, substrate concentration and agitation sources in the production of lauric acid or biotransformation study.

Table 4.7: Factors and levels in the lauric acid production optimization study

Factor	Optimization parameters	Unit	Level 1	Level 2	Level 3	Level 4	Level 5
A	Temperature	^{o}C	25	30	35	40	45
B	pH	-	5	6	7	8	9
C	Substrate Concentration	%	1.0	2.0	3.0	4.0	5.0
D	Agitation	RPM	50	75	100	125	150

Taguchi method designed an orthogonal array of 25 experiments (L_{25}) arranging the four input factors and corresponding levels in a crisscross way. These 25 experiments were performed under different culture condition which was designed by software. Coconut oil was taken as carbon source or substrate and each experiment was carried out for 8 days to check the maximum biomass and lauric acid. Microbial biomass (g/l) and lauric acid (%) were taken for consideration and results of L_{25} experiments and their signal to noise ratio (S/N ratios) are given in Table 4.8.

4.4.1.2 Normalization of experimental data:

From the results of L_{25} (5^4) orthogonal arrays are reported in Table 4.8. Two procedures were adopted to obtain GRG (γ) as shown in Table 4.10. The response graph is based on the average values of the grey relational coefficient. Accordingly optimal conditions were selected from the response graph. The raw data of microbial biomass at different levels for specific factor are responsible in Table 4.8. Normalized data and normalized function of GRA (based on longer-the-better) as per *Equation 1* is summarized in Table 4.9. Grey relational coefficients ($\xi_i(k)$) and their grey grades was obtained through *Equation 2* and *Equation 3* to evaluate the multiple performance characteristics, respectively.

Table 4.8: S/N ratios of Biomass and lauric acid concentration obtained in L_{25} design

Expt No.	Factors				Biomass Conc (g/l) (VBP-01)	Lauric acid (%) (VBP-01)	S/N ratio of biomass (VBP-01)	S/N ratio of lauric acid (VBP-01)
	A	B	C	D				
1	25	5	1	50	0.0751	0.9874	-8.2010	26.0249
2	25	6	2	75	0.1405	0.9698	-5.4818	26.5472
3	25	7	3	100	0.1954	0.9324	-3.7150	27.5643
4	25	8	4	125	0.0961	0.9769	-7.2302	26.3404
5	25	9	5	150	0.0000	0.9150	-12.9563	25.6298
6	30	5	2	100	0.3185	0.7717	-0.7148	30.9383
7	30	6	3	125	0.4018	0.6524	0.8515	32.7997
8	30	7	4	150	0.3890	0.7028	0.6282	32.0607
9	30	8	5	50	0.2554	0.8425	-2.1248	29.6088
10	30	9	1	75	0.2238	0.8084	-2.9260	30.2750
11	35	5	3	150	0.4256	0.5696	1.2516	33.8903
12	35	6	4	50	0.5053	0.3564	2.4705	36.1952
13	35	7	5	75	0.4728	0.4630	1.9936	35.1190
14	35	8	1	100	0.5661	0.2209	3.2989	37.3952
15	35	9	2	125	0.5437	0.3169	3.0028	36.5629
16	40	5	4	75	0.6989	0.2414	4.8707	37.2247
17	40	6	5	100	0.8046	0.1158	5.9465	38.2242
18	40	7	1	125	0.8220	0.1023	6.1113	38.3249
19	40	8	2	150	1.0000	0.0000	7.6403	39.0539
20	40	9	3	50	0.7945	0.1596	5.8496	37.8885
21	45	5	5	125	0.3657	0.6267	0.2060	33.1526
22	45	6	1	150	0.4412	0.4586	1.5036	35.1661
23	45	7	2	50	0.5149	0.4019	2.6067	35.7520
24	45	8	3	75	0.4513	0.4897	1.6629	34.8278
25	45	9	4	100	0.3954	0.5480	0.7406	34.1534

Table 4.9: Response table for Grey relational analysis

Experiment No.	Normalized values(VBP-01)		G R Coefficient(VBP-01)	
	Biomass	**Lauric acid**	**∑ Biomass**	**∑ Lauric acid**
1	0.0751	09874	0.3509	0.9754
2	0.1405	0.9698	0.3678	0.9431
3	0.1954	0.9324	0.3833	0.8809
4	0.0961	0.9769	0.3562	0.9558
5	0.0000	0.9150	0.3333	0.8547
6	0.3185	0.7717	0.4232	0.6865
7	0.4018	0.6524	0.4553	0.5899
8	0.3890	0.7028	0.4501	0.6272
9	0.2554	0.8425	0.4017	0.7605
10	0.2238	0.8084	0.3918	0.7230
11	0.4256	0.5696	0.4654	0.5374
12	0.5053	0.3564	0.5026	0.4372
13	0.4728	0.4630	0.4867	0.4822
14	0.5661	0.2209	0.5324	0.3909
15	0.5437	0.3169	0.5229	0.4226
16	0.6989	0.2414	0.6241	0.3973
17	0.8046	0.1158	0.7190	0.3612
18	0.8220	0.1023	0.7374	0.3577
19	1.0000	0.0000	1.0000	0.3333
20	0.7945	0.1596	0.7087	0.3730
21	0.3657	0.6267	0.4408	0.5725
22	0.4412	0.4586	0.4722	0.4801
23	0.5149	0.4019	0.5075	0.4553
24	0.4513	0.4897	0.4768	0.4949
25	0.3954	0.5480	0.4527	0.5252

4.4.1.3 Grey Relation Grade (GRG) Calculation:

GRG is the overall representative of both the responses. The multi response optimization problem was transformed into a single response by using this approach. S/N ratio and mean values obtained from the GRG values were shown in Table 4.10.

Table 4.10: GRG ranking, mean and S/N ratio of GRG values

Expt. No	GRG (γ) (VBP-01)	S/N Ratio (VBP-01)	Mean (VBP-01)	Grey ranking (VBP-01)
1	0.663143	-3.56786	0.663143	2
2	0.655423	-3.66956	0.655423	4
3	0.632082	-3.98452	0.632082	5
4	0.655996	-3.66198	0.655996	3
5	0.593992	-4.52438	0.593992	6
6	0.554859	-5.11634	0.554859	9
7	0.522586	-5.63685	0.522586	14
8	0.538626	-5.37426	0.538626	13
9	0.581118	-4.71471	0.581118	7
10	0.557369	-5.07714	0.557369	8
11	0.501392	-5.99645	0.501392	17
12	0.469937	-6.55920	0.469937	24
13	0.484455	-6.29493	0.484455	20
14	0.463161	-6.68536	0.463161	25
15	0.472734	-6.50766	0.472734	23
16	0.510680	-5.83702	0.510680	15
17	0.540101	-5.35049	0.540101	12
18	0.547583	-5.23101	0.547583	10
19	0.666667	-3.52183	0.666667	1
20	0.540871	-5.33812	0.540871	11
21	0.506666	-5.90556	0.506666	16

22	0.476179	-6.44459	0.476179	22
23	0.481446	-6.34904	0.481446	21
24	0.485821	-6.27047	0.485821	19
25	0.488940	-6.21488	0.488940	18

The experimental trial number 19 of *Pseudomonas aeruginosa (VBP-01)* was having higher grey relational grade, 0.666667 than other experimental trials and the experimental trial number 14 was having grey relational grade, 0.463161. Normally, the larger the grey relational grade, the closer the product quality to the ideal value.

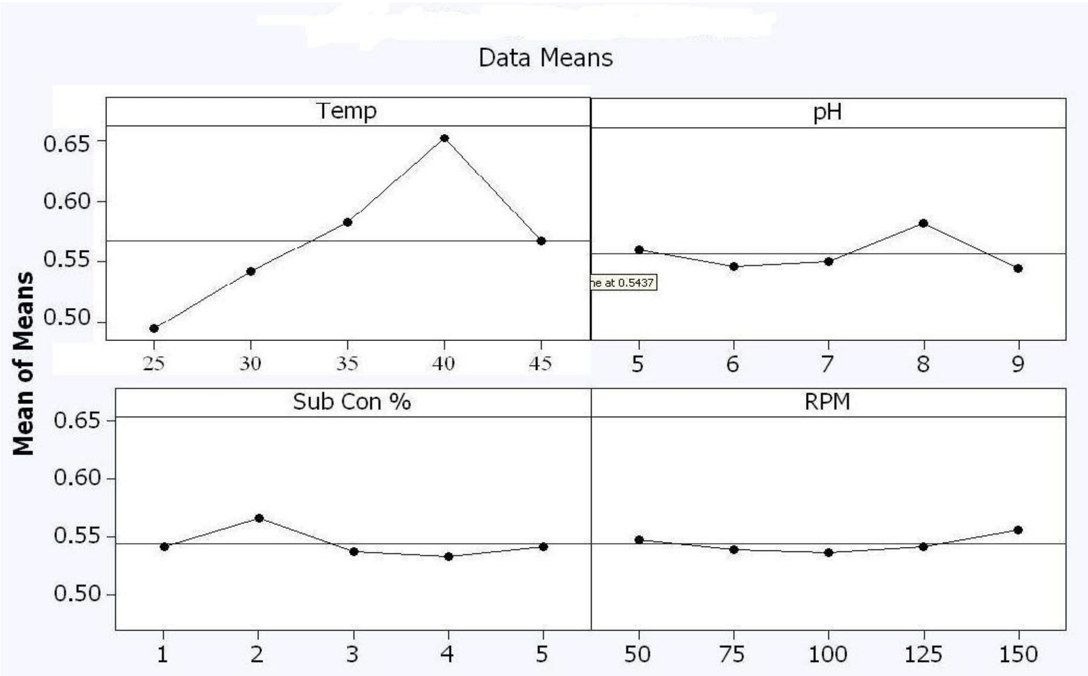


Figure 4.14: *Main Effect Plot for means of Pseudomonas aeruginosa (lauric acid production)*

The optimum process parameters required to yield higher biomass and produce maximum lauric acid by *Pseudomonas aeruginosa* at optimum process parameters such as Temperature-40^0C, pH-8, Substrate concentration-2% and Agitation (RPM) – 150 is reported in figure 4.14.

Normal probability plot is used as an informal means of assessing the non-normality of a set of data. It is a graphical representation of assessing whether the data set is approximately normally distributed or not. It is also basically a plot of the ordered observations from a sample against the corresponding percentage points from the standard normal distribution [web ref. 14]. The normal probability plot of our study is shown in Figure 4.15.

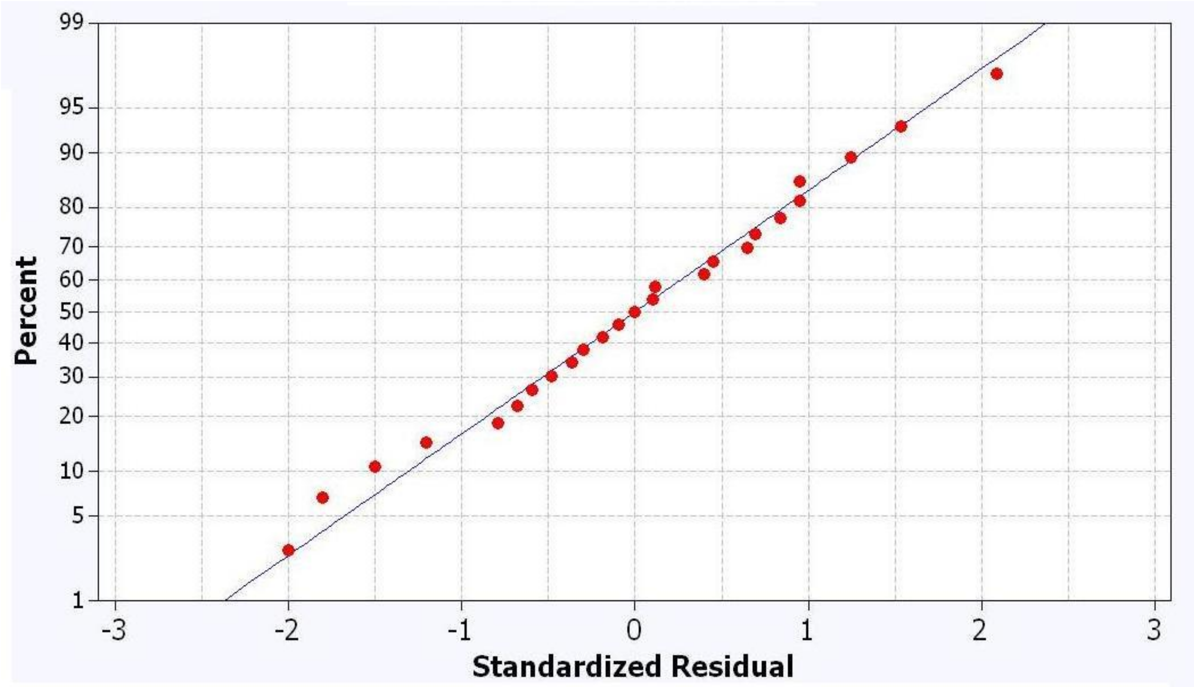


Figure 4.15: *Normal probability plot for means of Pseudomonas aeruginosa (Lauric acid production)*

4.4.1.4 ANOVA calculation for Pseudomonas aeruginosa (lauric acid production):

Using GRG value, ANOVA was designed to identify the significant factor. The results of ANOVA were presented in Table 4.11. Predominance of temperature 60^0C on maximum *Pseudomonas aeruginosa* biomass growth and higher percentage of lauric acid production is clearly represented. Subsequently pH-8, substrate concentration- 2% and agitation (RPM) showed their responses in decreasing order. The mean of these response graph based on GRG with four variables and levels illustrated in Figure 4.14. The standard deviation for our optimization study was 0.03760. This study using Grey based Taguchi enables to understand the influence of four different process parameters and their variation on the growth of bacterial biomass and lauric acid production efficiency of *Pseudomonas aeruginosa.*

Table 4.11: ANOVA table of *Pseudomonas aeruginosa*

Source	DF	Seq SS	Adj SS	Adj MS	F	% Contri.	Rank
Temperature	4	0.085259	0.085259	0.021315	15.0	80.01	1
pH	4	0.005331	0.005331	0.001333	0.94	5	2
Sub. Conc(%)	4	0.003437	0.003437	0.000859	0.61	3.22	3
RPM	4	0.001212	0.001212	0.000303	0.21	1.13	4
Residual error	8	0.011309	0.011309	0.001414	-	10.61	-
Total	24	0.106548	-	-	-	100	-

4.5 Production of Lauric acid:

4.5.1 Lauric acid production by lab scale fermenter:

As biotransformation reaction requires the presence of a suitable biological catalyst which can consume substrate and convert it into our desired product. Lab scale bio fermenter was applied in our study by increasing volume from 100 ml shaker flak to 1 litre volume. The bacterial whole cell was used as a catalyst for lauric acid production. The lab scale fermenter which was used for this study was shown in figure 4.16.

Figure 4.16: *Fermenter set-up used for lauric acid production, IIC Bio fermenter*

The optimum conditions which were obtained from optimization study were used as process parameter for lauric acid production in anaerobic condition. Anaerobic condition was maintained by purging nitrogen gas into the reactor and the level of oxygen was measured by DO sensor and visually detected by methylene blue as explained in materials and methods chapter.

Production process was carried out for 8 days. The sample was drawn from the fermenter by sample isolation port with sterile condition on daily basis up to 8 days to know the amount of lauric acid produced with respect to time. The sample which was drawn from the fermenter and mixed with organic solvent (*n*-hexane) is a ratio of 3:7 to collect and detect the fatty acid (major part is lauric acid in our study). It was collected and dissolved in organic solvent was subjected to GC-MS analysis for quantitative analysis. Organic solvent was used to dissolve the sample for the ease detection of gas chromatography. The GC analysis graphs were shown in figure 4.17 starting from first day to eight day in chronological order. In GC graph the target product of our study was rounded.

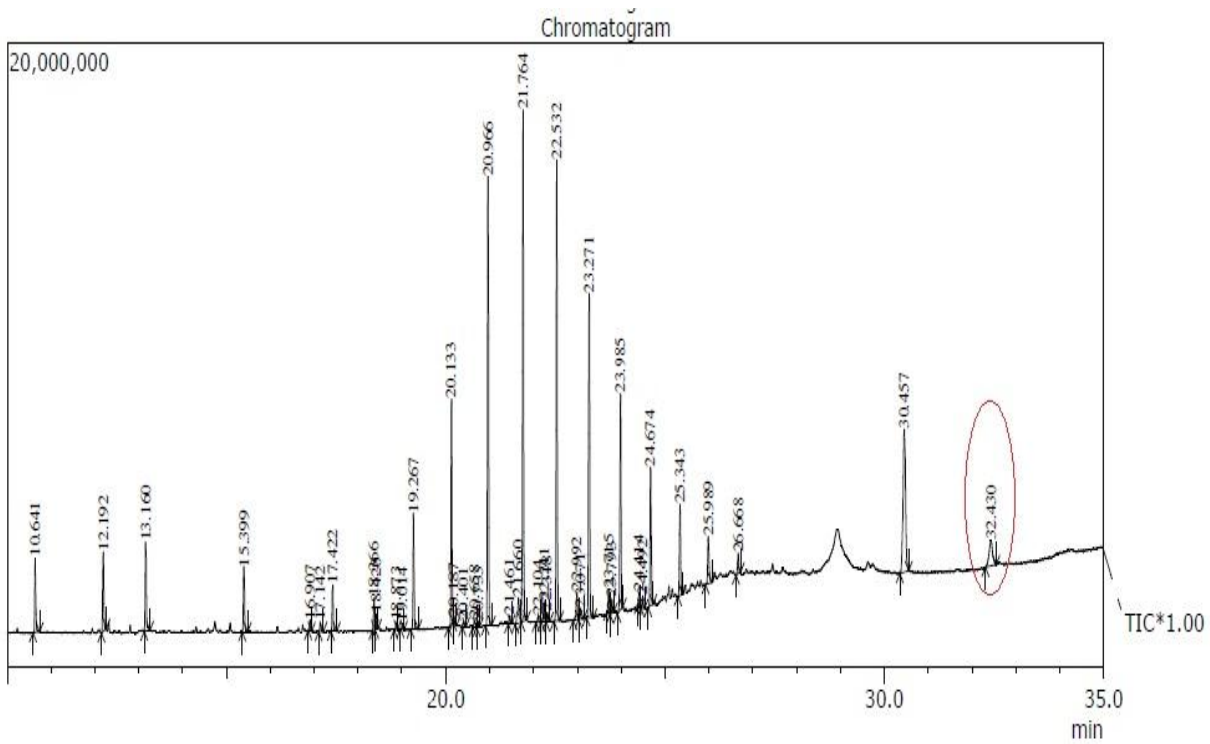


Figure 4.17: *Lauric acid production in Fermenter (Day-1)*

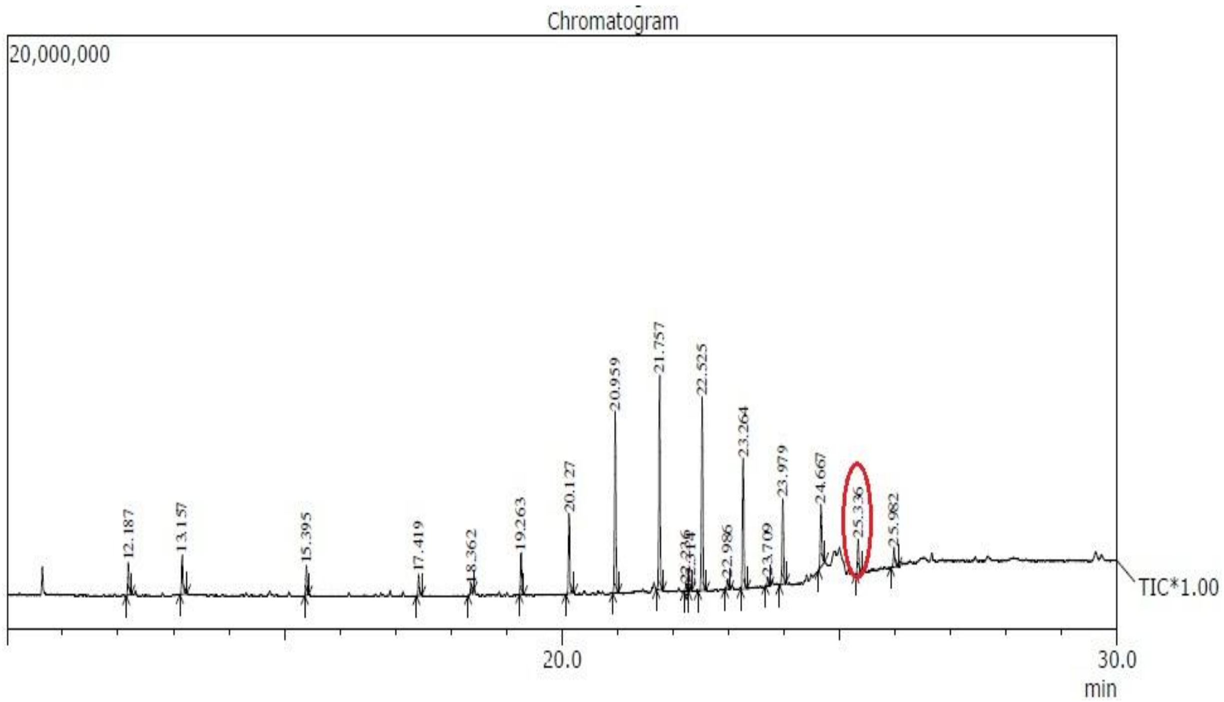


Figure 4.18: *Lauric acid production in Fermenter (Day-2)*

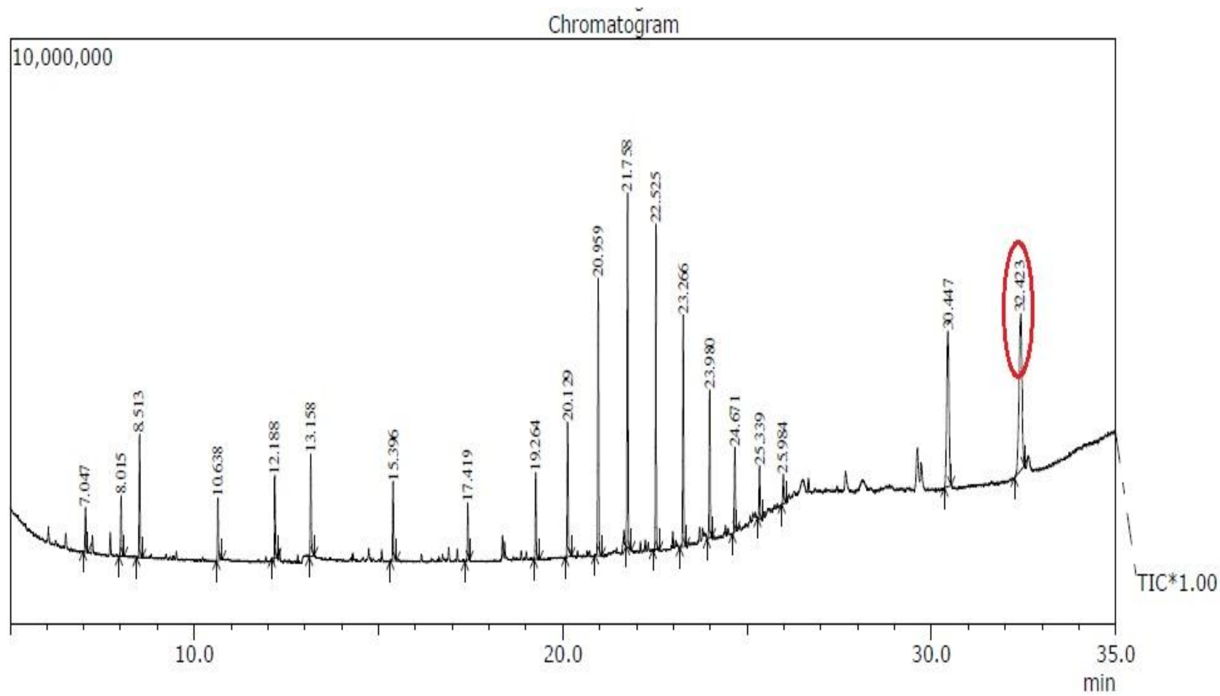


Figure 4.19: *Lauric acid production in Fermenter (Day-3)*

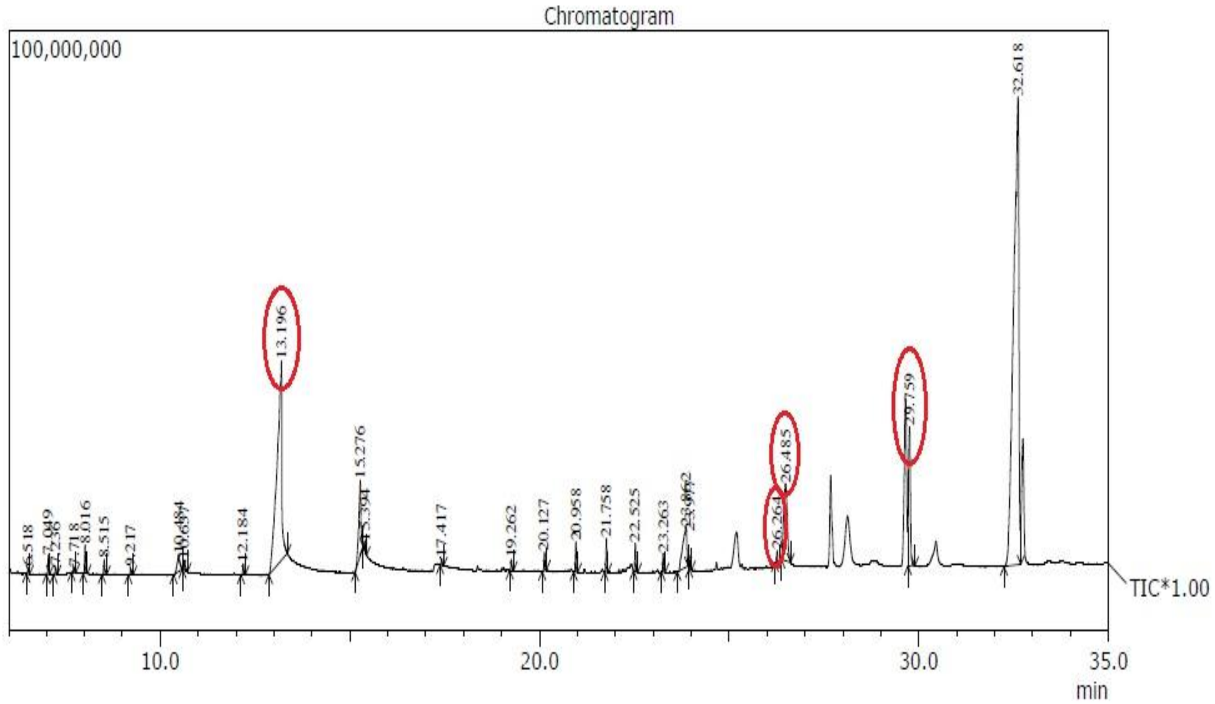


Figure 4.20: *Lauric acid production in Fermenter (Day-4)*

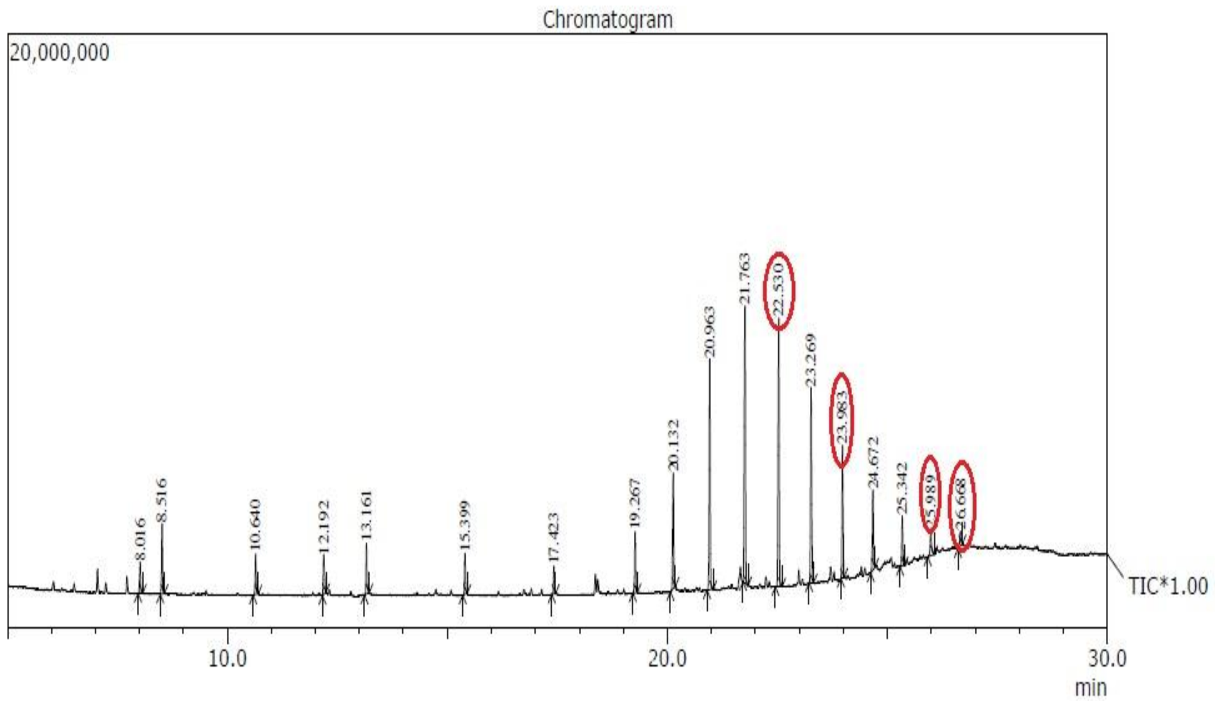


Figure 4.21: *Lauric acid production in Fermenter (Day-5)*

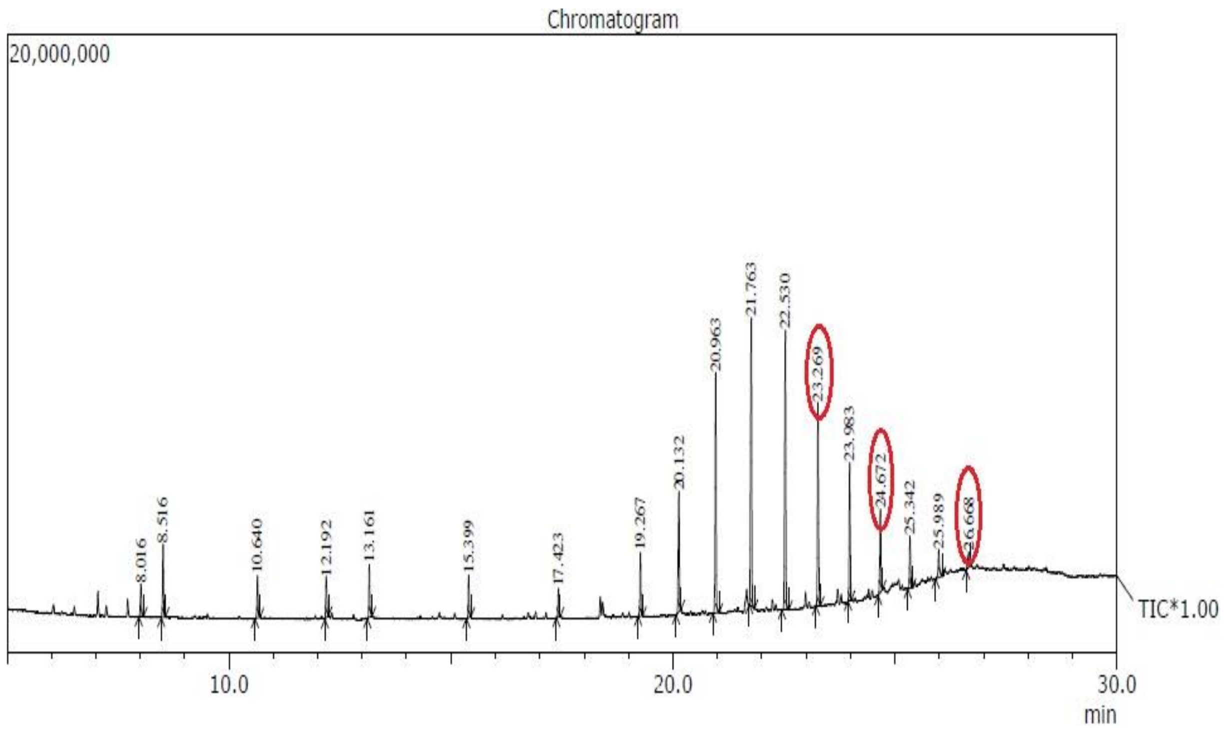


Figure 4.22: *Lauric acid production in Fermenter (Day-6)*

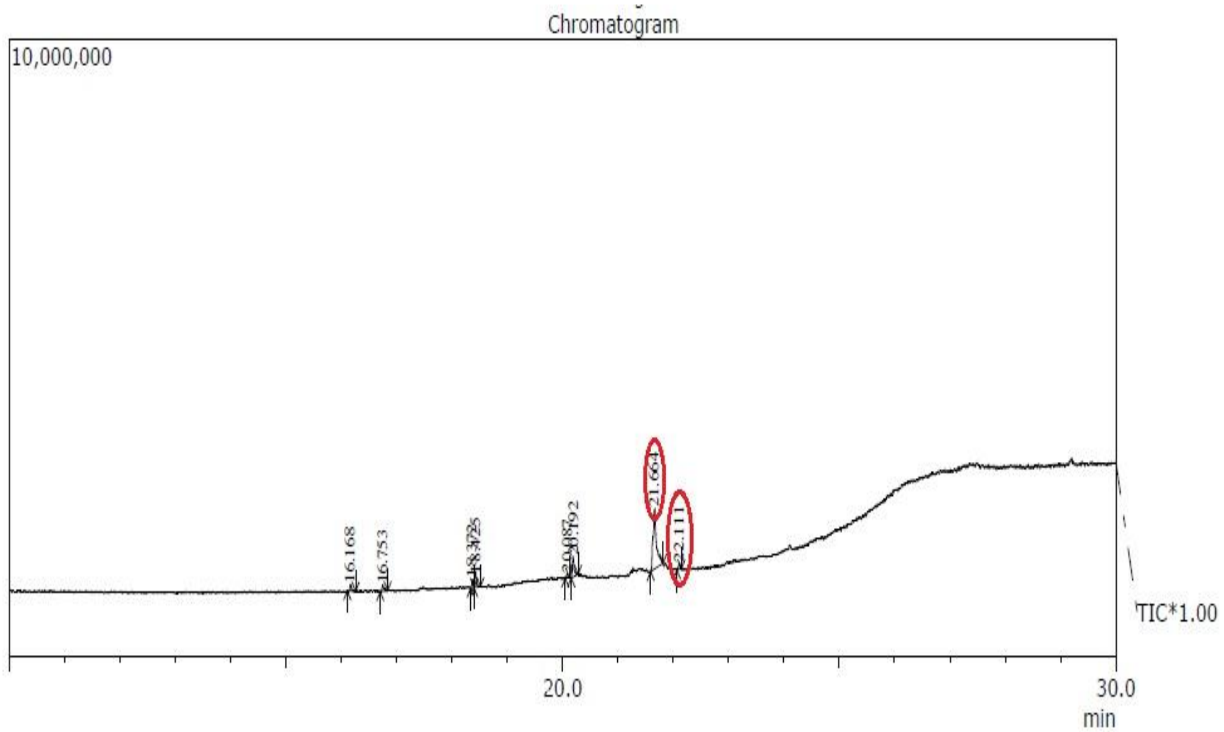


Figure 4.23: *Lauric acid production in Fermenter (Day-7)*

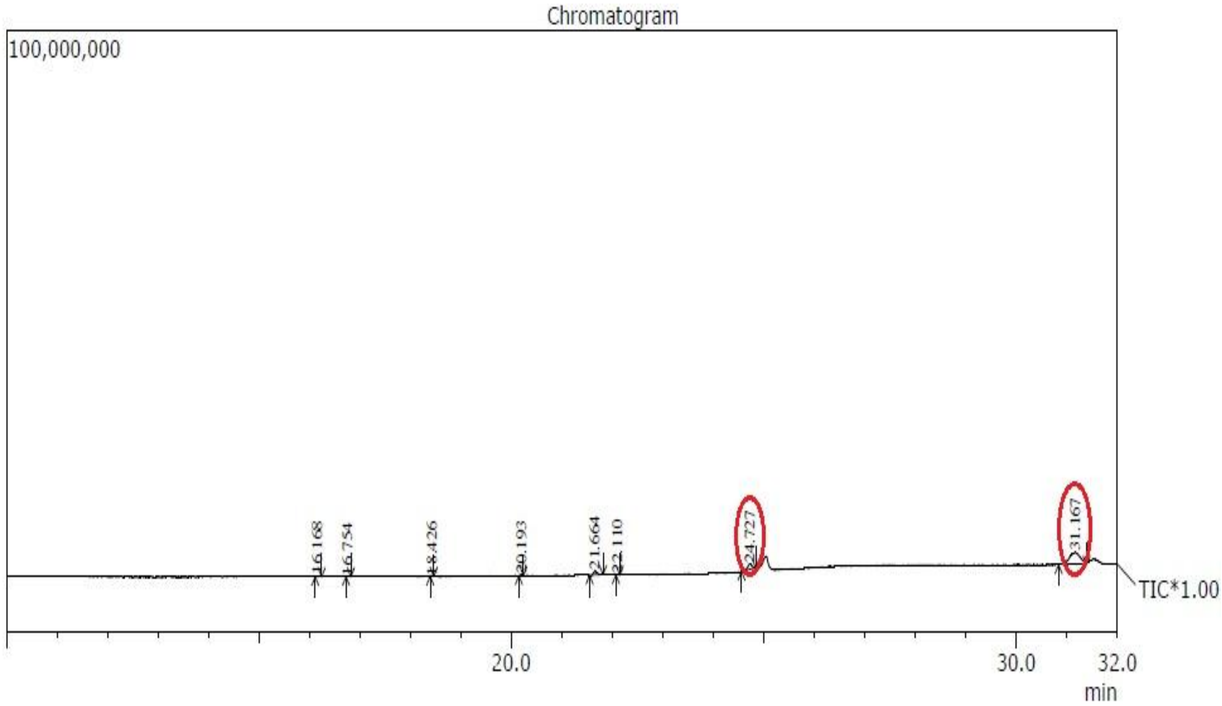


Figure 4.24: *Lauric acid production in Fermenter (Day-8)*

Using GC graph the quantity of lauric acid produced daily for eight days was calculated and represented in Table 4.12. The formula for calculation of compound percentage can be found in Appendix-L.

Table 4.12: Day to day analysis of lauric acid (by GC-MS method)

Day	Lauric acid (%)
1	2.14
2	14.09
3	15.89
4	33.11
5	36.56
6	51.98
7	67.29
8	89.68

On first day, there was no significant development in the production of medium chain fatty acid (lauric acid) due to adaptation time of microbes to the particular environment. In second and third day the lauric acid percentage was nearly same but percentage was more as compared to the initial day. The lauric acid (C12:0) produced, represented 33.11%, 36.56% and 51.98% of total fatty acids, respectively. The hydrolysis (biotransformation) reaction activity was increased significantly between 3rd and 7th days. Although microbes have reached in the stationary phase but due to formation of microbial lipase, helps in splitting of oil into glycerol and fatty acid. In anaerobic condition glycerol was up taken by the microbes as a carbon source. On 8th day, the production of lauric acid (C12:0) was increased up to 89.68%. while glycerol, glycerol trilaurate and residual oil were found in traces.

Hence, this study revealed that microbial transformation of coconut fat increased the percentage of lauric acid (MCSFA) as compare with other fatty acids. These results were compared with the Codex Alimentarius Commission International Standard value [Young, 1983]. Our results are in good agreement with those reported by Gopala Krishna et. al. 2010, who suggested a concentration of 62% lauric acid in fatty acids obtained by enzymatic hydrolysis from coconut oil and some other vegetable oils. Substrate and product kinetics of oil and lauric acid were shown in figures 4.25-4.27.

4.6 Substrate and Product kinetics:

4.6.1 Substrate Kinetics (Aerobic & Anaerobic):

it was clearly observed (Figures 4.25 and 4.26) that during aerobic and anaerobic condition break down of oil follows first order kinetics which can be explained in terms of pseuedo first order kinetics of hydrolysis of oil into the fatty acid and glycerol by enzyme lipase. The concentration versus time plot for both aerobic and anaerobic condition is represented in figure 4.25 and 4.26 respectively. The equation based on first order kinetic model under aerobic and anaerobic condition is mentioned in *Equation* 4 & 5 respectively.

Aerobic model equation for oil breakdown: $1.1947e^{(-0.2t)}$ (4)

Anaerobic model equation for oil breakdown: $0.999+0.188e^{(-0.2573t)}$ (5)

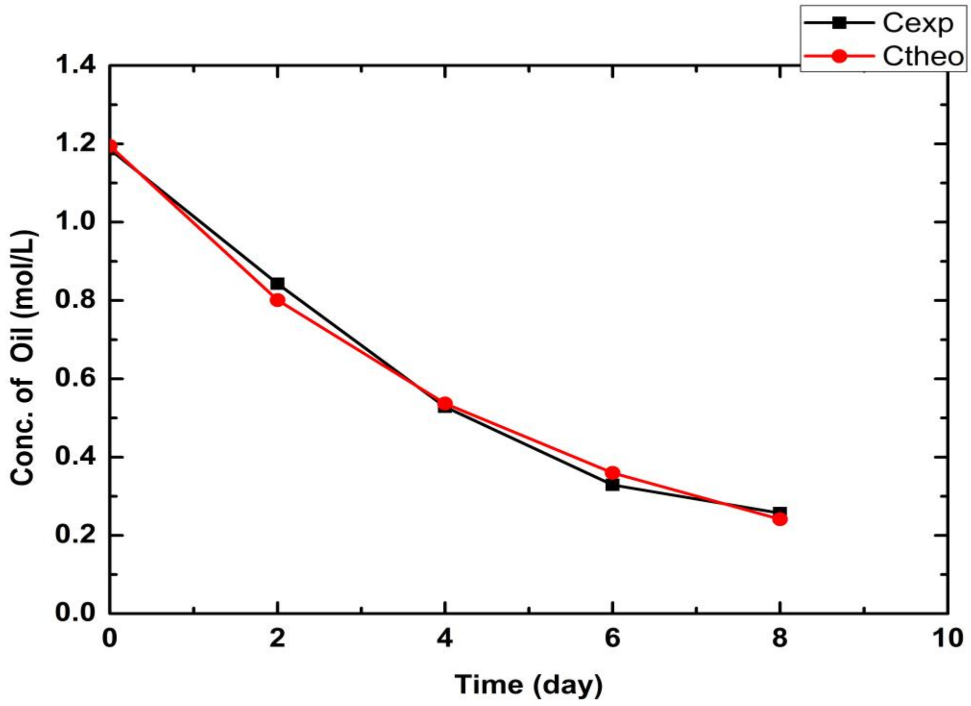


Figure 4.25: *Concentration versus Time curve (Aerobic)*

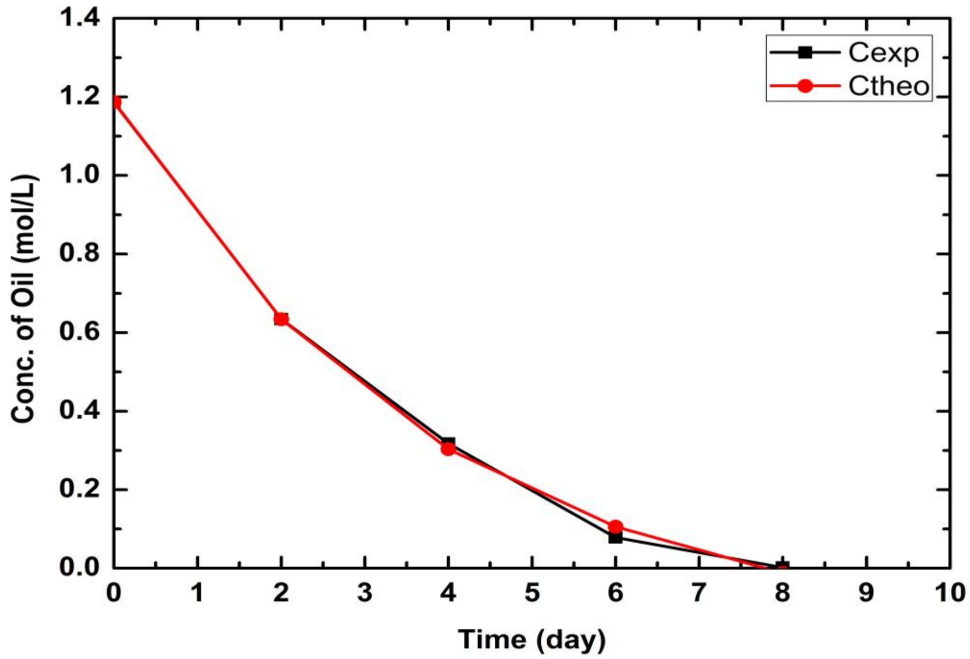


Figure 4.26: *Concentration versus Time curve (Anaerobic)*

It can be clearly observed that oil was completely degraded in anaerobic condition within 8 days where as only 75% oil could be degraded under aerobic condition in the same period. breakdown occurs effectively. Hence further product kinetics was studied.

4.6.2 Product kinetics:

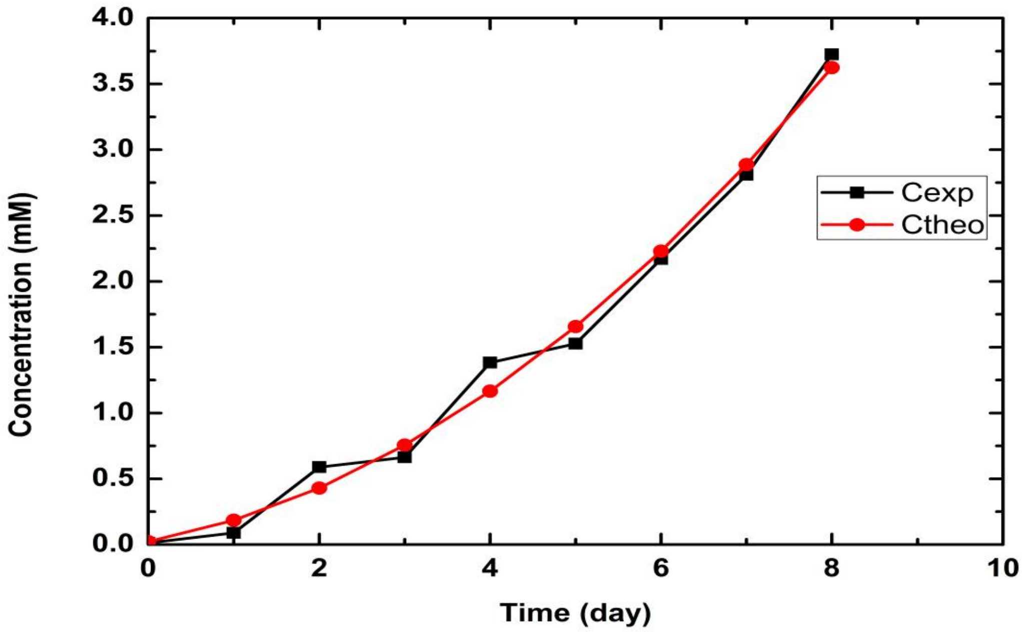


Figure 4.27: *Concentration versus Time curve (Anaerobic)*

The target product of our study was the production of lauric acid from coconut oil. The product kinetics focuses on the production of lauric acid. From the above figure 4.27 the curve was modelled to fit second order kinetics. The empirical equation for the formation of lauric acid based on time (days) is given in *Equation* 6.

Concentration of lauric acid (C_{lauric}) = $0.0411t^2 + 0.1214t + 0.0225$ (6)

It was observed that the experimental results were in good agreement to the theoretical values (Figure 4.27).

4.6.3 Selection of reactor type for the production of lauric acid:

Continuous stirred tank reactor (CSTR) is preferred to plug flow reactor (PFR) for breakdown of oil using *Pseudomonas aeruginosa* microbe to produce lauric acid. The area under the curve gives the effective liquid volume of the reactor to be desired[Figure

2.9](which in this case say for 1000ltr/day feed rate the volume of the CSTR was calculated to be 4375 ltrs with the space time(τ) of 3.5 days. The space time for PFR is 11.5 days. So, CSTR is preferred than PFR for lauric acid production of lauric acid.

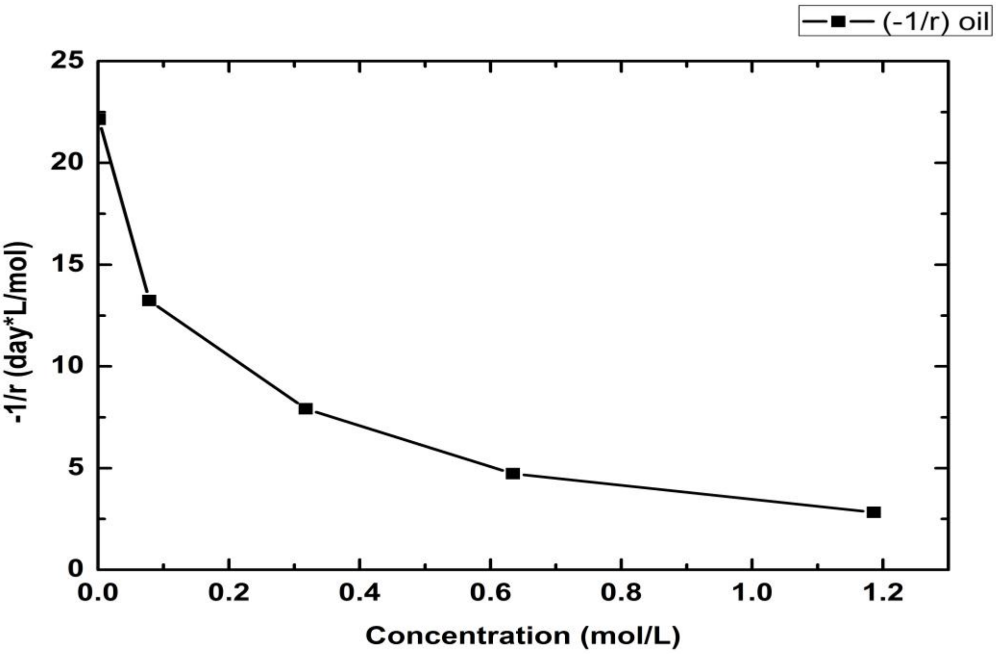


Figure 4.28: *Rate of reaction versus Concentration curve for oil break down*

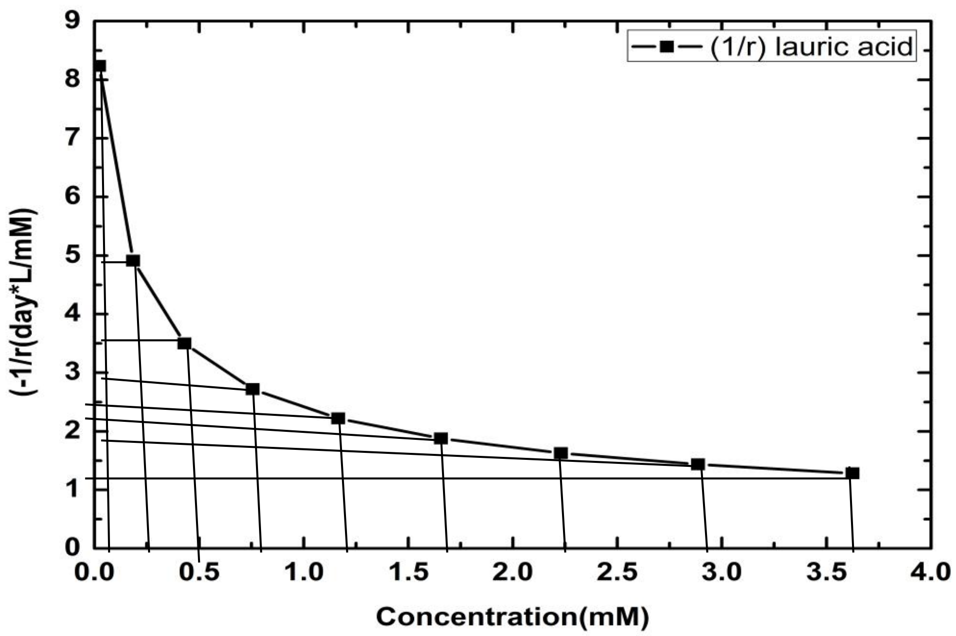


Figure 4.29: *Rate of reaction versus Concentration curve for lauric acid production*

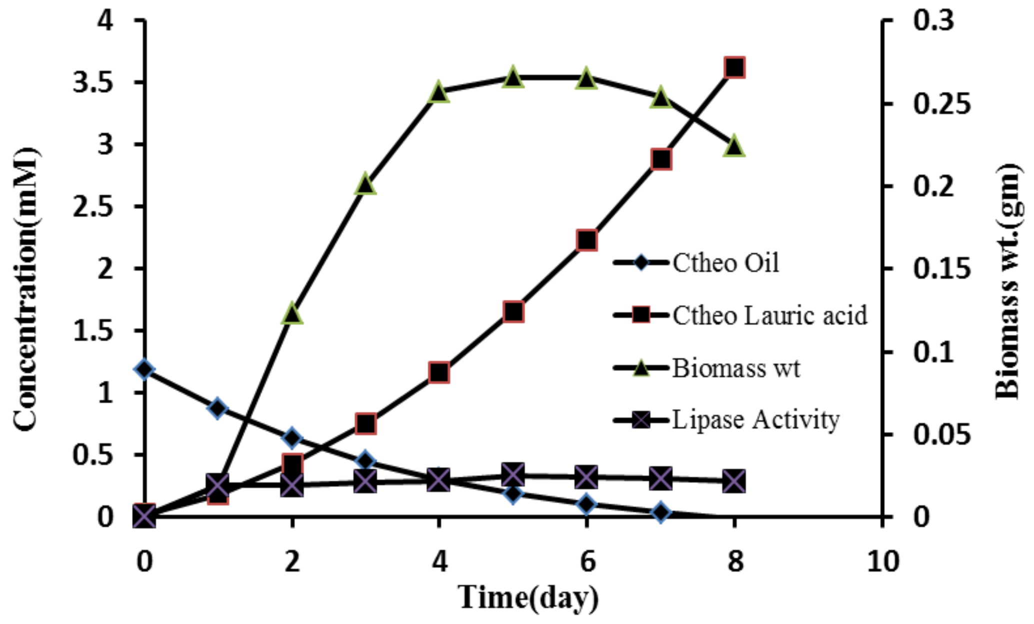


Figure 4.30: *Relation between substrate and product w.r.t. time*

Substrate degradation occurs when oil present in the solution disappeared. The relation (Figure 4.30) shows that when maximum degradation of oil occurs, the rate of formation of formation of lauric acid is high. The lauric acid production occured high until unless the substrate is available. Similarly, higher the rate of formation of biomass while the lipase activity present in the solution. Even though microbes entered into the stationry phase the formation of lauric acid production was increasing due to the presence of lipase enzyme which is responsible for the splitting of oil .

4.7 Basic Mechanism of Biotransformation in this study:

Triacylglycerol, the main component of natural oil is stepwise converted into diacylglycerol, monoacylglycerol and glycerol by hydrolysis accompanied with the liberation of a fatty acid at each step [Beisson 2000]. Lipases (triacylglycerol acyl hydrolases, E.C. 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial potential. Lipases catalyze the hydrolysis of triacylglycerol to glycerol and free fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an oil–water interface [Martinelle et.

al. 1995]. Lipases are serine hydrolases. Microbial enzymes are often more useful than enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media. Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer [Wiseman A. 1995].

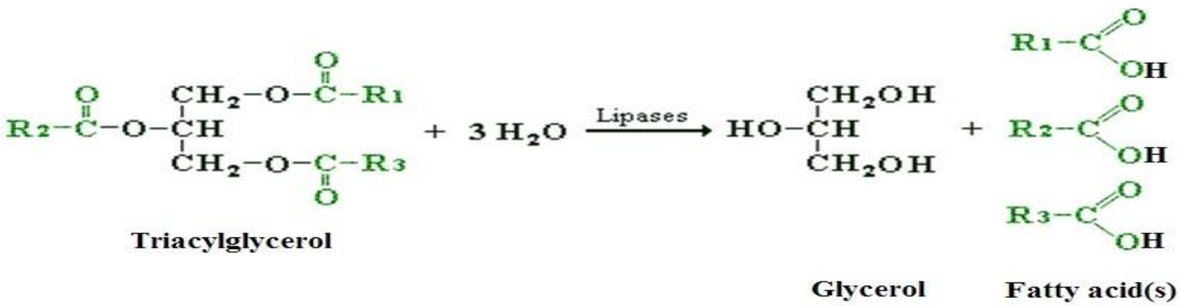


Figure 4.31: *The hydrolyse by lipases of triacylglycerol in glycerol and fatty acids*

In our study the biotransformation process has been carried out by two processes i.e. in aerobic and anaerobic process condition. So the basic mechanism for aerobic and anaerobic biotransformation was previously reported. The detail of basic mechanism for both the process is described below:

Microbe is producing the enzyme lipase for the splitting of triacylglycerol to glycerol and fatty acid by hydrolysis reaction. The majority of enzymes in industrial use are extracellular proteins from bacterial or fungal sources and it is easier and therefore cheaper to use such an enzyme for commercial use. The conformation of enzyme can be done by various enzyme detection methods which is available in various literatures. Under aerobic condition lipase produced hydrolysis triacylglycerides to fatty acid and glycerol. The fatty acid is then incorporated by the microbes which break down fatty acids via β-oxidation pathway (Figure 4.32) [Marino 1998] leaving excess glycerol in the system due to easily available oxygen and carbon source.

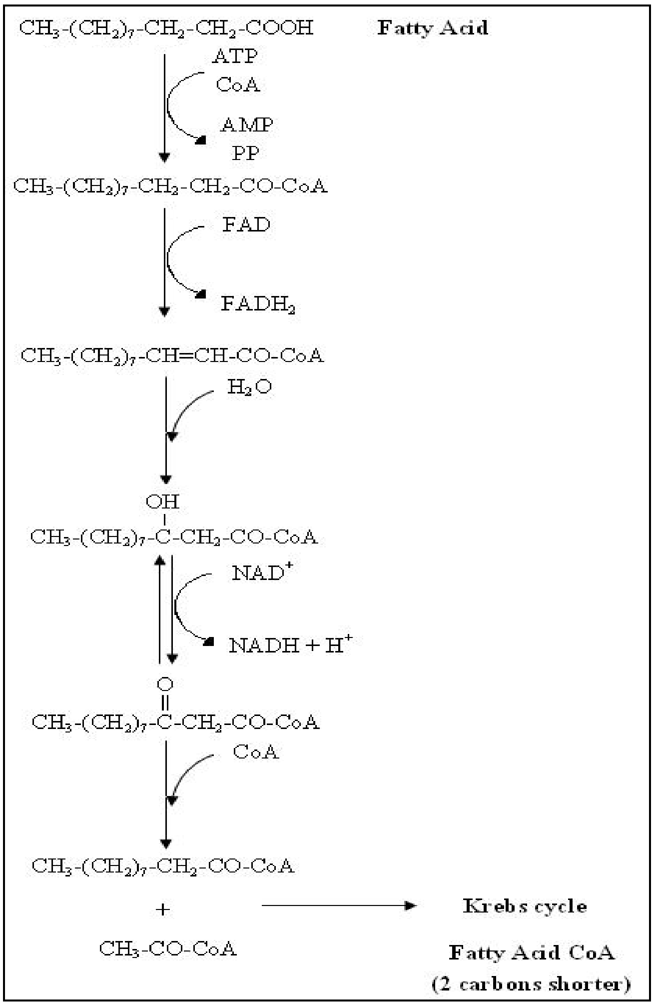


Figure 4.32: *β-oxidation metabolic path way in aerobic [Marino, 1998]*

But in anaerobic condition the presumed pathway is little bit complex. Here the glycerol metabolism follows two alternative pathways leading to the primary glycolysis pathway.

Route-1: Follows glycerol reduction to glyceraldehyde which ends with glycerate 3-Phosphate; the intermediate product of glycolysis pathway.

Route-2: Follows glycerol to glycerone as it is oxidized which ends with glyceraldehyde 3-phosphate Because of microbes not getting sufficient amount of oxygen for its metabolism, it uses an alternate metabolic pathway to hydrolyse oil to glycerol and fatty acids. Due to oxygen demand favouring β oxidation, microbes utilize glycerol for its metabolism leaving free fatty acid in the system.

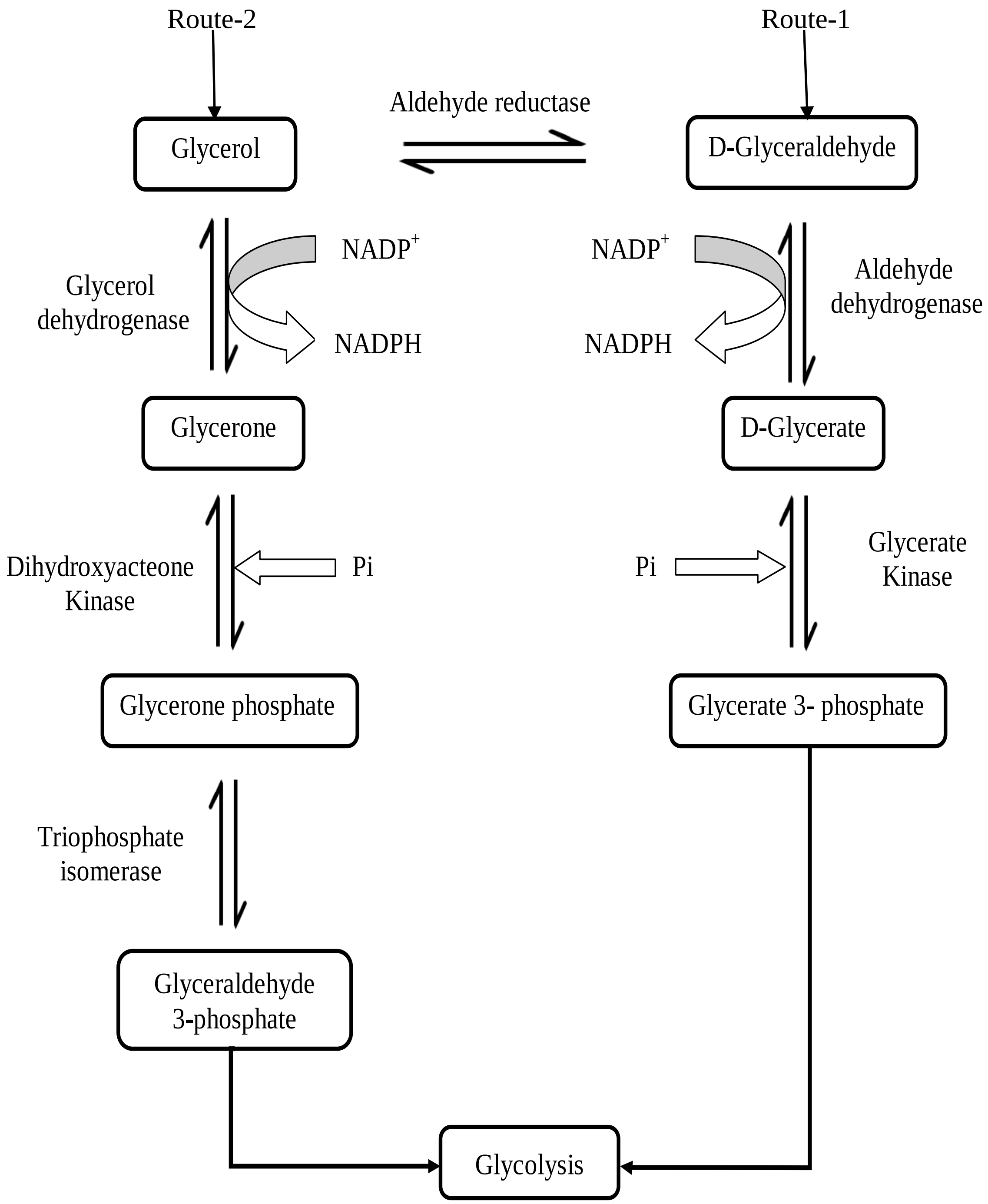


Figure 4.33: *Pathway for Glycerol Metabolism(Anaerobic)[Kegg pathways]*

4.8 Lipase Enzyme activity:

4.8.1 Detection of Lipase enzyme:

It was possible to visualize lipase-producing microbial colonies on solid media depending on the used lipase substrate. This type of detection method was having several advantages like the elimination of the requirement of specific dyes that can reduce the cost of analysis and prevent the inhibition of microbial growth as microorganisms may be sensitive to dyes (Thomson, et al. 1999). From literature review we have found that the Pseudomonas sp. produces extracellular lipase enzymes which help in splitting of oil into glycerol and fatty acid [Chang et al. 2007]. In our study the lipase enzymes were detected by Substrate agar plate method and Rhodamine-B dye agar plate method which were shown in Figure 4.34 and 4.35.

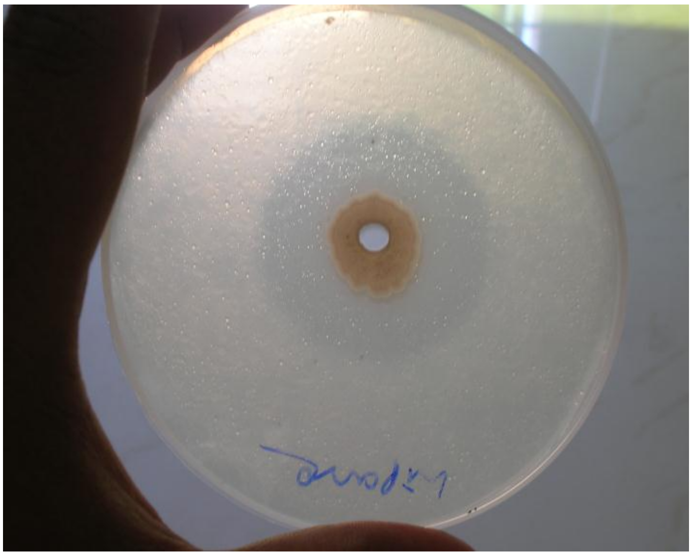


Figure 4.34: *Detection of Lipsae by Substrate agar plate method*

Diffusion assay was used to identify the presence of extracellular microbial lipase in our study. In this method, a growth medium with tributyrin(1%) was prepared and was solidified with agar. 100 μl of the microbial culture supernatant was subjected into the well. It can be seen that the clear zone around the colonies after incubation was formed (Figure 4.34). The formation of clear zone was due to hydrolysis of substrate and shows the presence of lipolytic activity by lipase enzyme [Thomson et al. 1999].

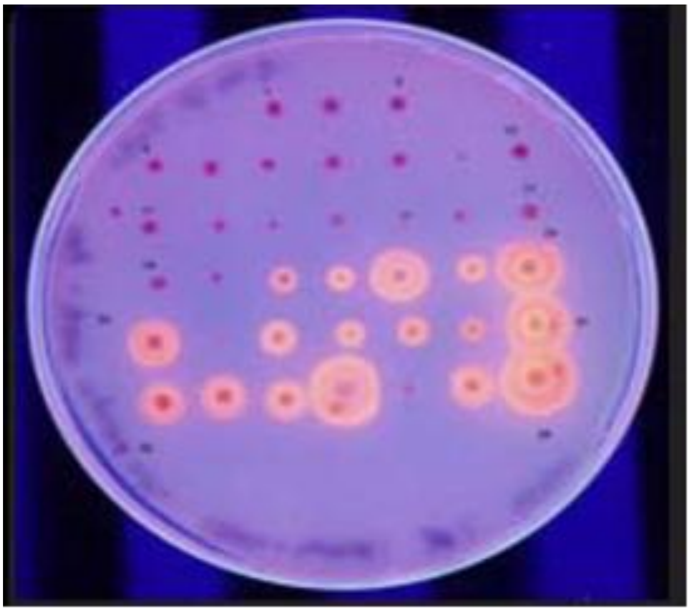


__Figure 4.35:__ Detection of Lipsae by Rhodamine-B dye agar plate method

Microbial lipase activity was identified by using fluorogenic dye Rhodamine-B in our study. Rhodamine B as an indicator reveals the presence of extracellular lipase. Coconut oil as lipid substrate was used in this study. Agar plates containing olive oil and rhodamine-B was opaque and pink colored. After incubation, Lipase producing bacteria forms orange fluorescent halos around their colonies under UV light, but lipase negative bacteria do not shows orange fluorescence upon UV irradiation. Distinguishable colour changes, as a result of pH changes that occurred due to the release of free fatty acids from triacylglycerol during lipolysis and formation of a rhodamine –B long chain fatty acid conjugates [Jaeger et. al. 2002].

4.8.2 Estimation of lipase activity:

The estimation of lipase activity was carried out using standard p-Nitrophenol method in our study. The standard graph for p-Nitrophenol was estimated and can be found in Appendix-D. The maximum amount of lipase was calculated. One unit of enzyme activity (IU) was defined as the amount of enzyme that liberated 1 µmol of p-nitrophenol per minute under the standard assay conditions. For determining the maximum activity of lipase in biotransformation process the sample was drawn on daily basis from aerobic and anaerobic condition for 192 hours. The lipase activity was estimated for each sample and plotted in graphs representing Time versus enzyme activity to determine the maximum lipase activity (Figure 4.36 & 4.37).

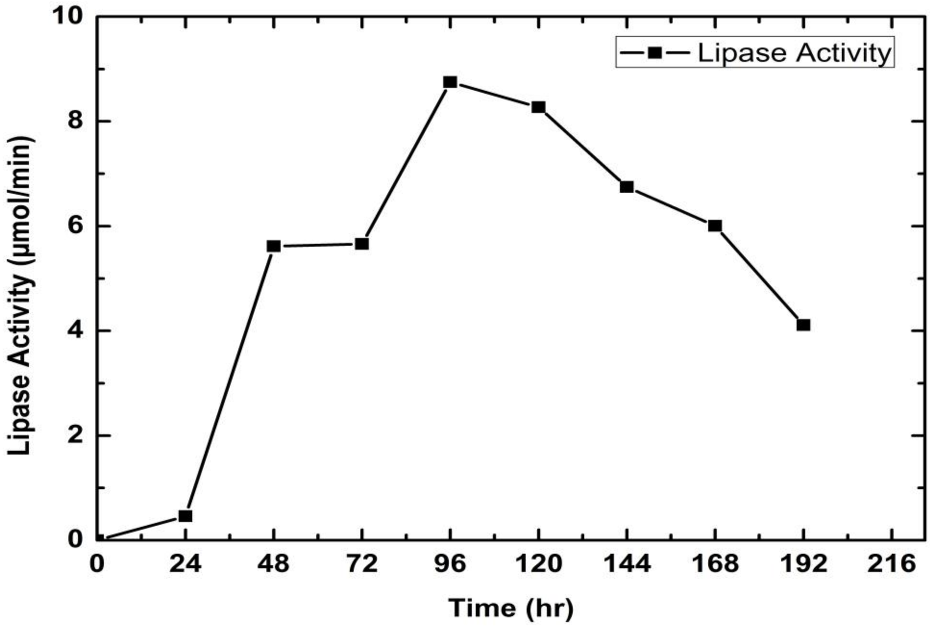


Figure 4.36: *Lipase activity in aerobic biotransformation process*

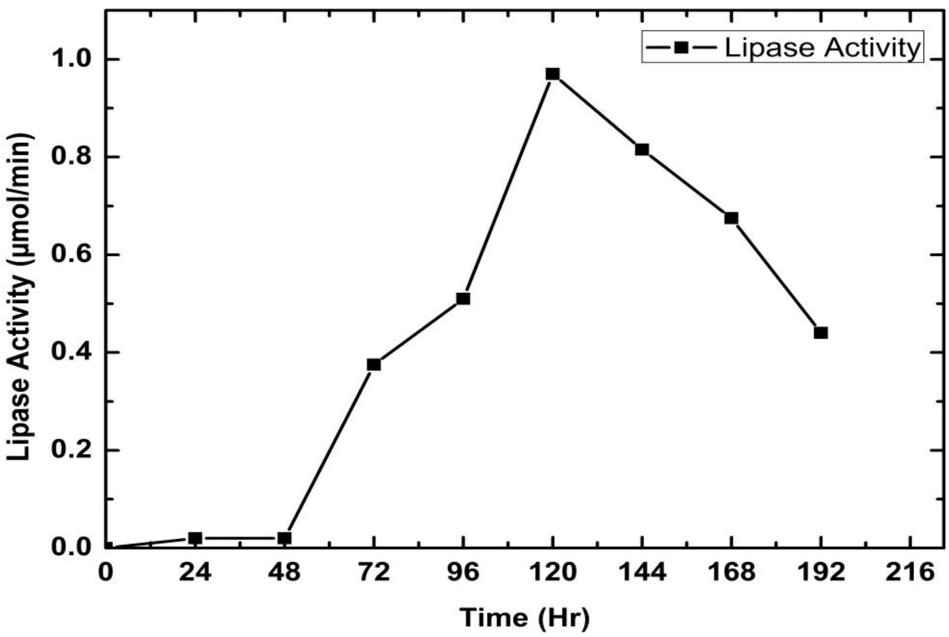


Figure 4.37: *Lipase activity in anaerobic biotransformation process*

From the figure 4.36 & 4.37 it can be seen that in aerobic condition maximum lipase activity 8.75 Unit was found in 96 hour where as in anaerobic condition maximum lipase activity was found 0.97 Unit was in 120 hours. Moreover aerobic condition was showing maximum lipase activity than compared to anaerobic condition. Microbe obtained their maximum energy during aerobic process that helped in respiration and completion of the metabolism process easily. Although it was able to survive in anaerobic condition but maximum energy could not be obtained. So as to survive and complete the metabolism process it produces very low amount of lipase by consuming low energy and obtained oxygen molecule from breaking down of substrate.

Due to its maximum activity aerobic condition was chosen for optimizing the maximum lipase activity.

4.8.3 Optimization of lipase activity:

Lipase activity optimization provides the optimum condition for maximum lipase activity so as to promote the reaction in our study. Optimization of the process parameters were carried out in conventional one factor at a time approach. The various important factors used for optimization study were Temperature, pH, Substrate concentration, Enzyme concentration (in terms of volume) and time of incubation [Sirisha et al. 2010]. Lipase activity optimization processes were carried out using standard p-Nitrophenol method. The above said process parameters were optimized and shown in various figures 4.38-4.42.

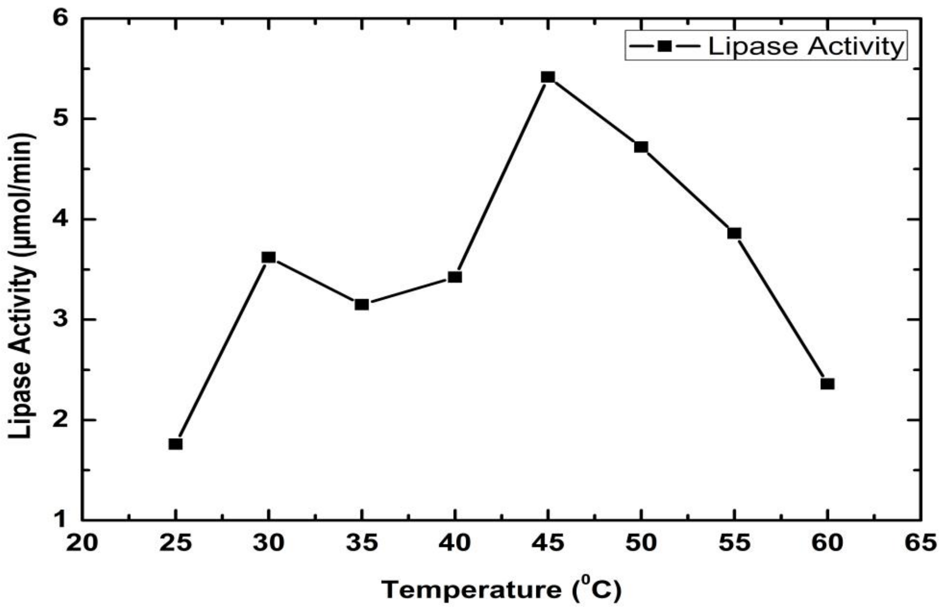


Figure 4.38: *Effect of temperature on lipase activity*

Temperature optimization was carried out by taking different level- 25ºC, 30ºC, 35ºC, 40ºC, 45ºC, 50ºC, 55ºC and 60ºC. The sample was incubated at different temperatures to determine the optimum temperature for maximum lipase activity. The optimum temperature for maximum lipase activity was measured at 45°C with 5.41 Unit (Figure 4.38). It is also depicted that enzyme can give its maximum activity at moderate temperature. Enzyme activity was reduced when the incubation temperature was increased above 45°C. At still higher temperatures the specific activity was reduced drastically due to denaturation of enzyme molecules [Tembhurkar et. al. 2010]. There was decrease in activity between 30^{0}C and 45^{0}C due to experimental variation.

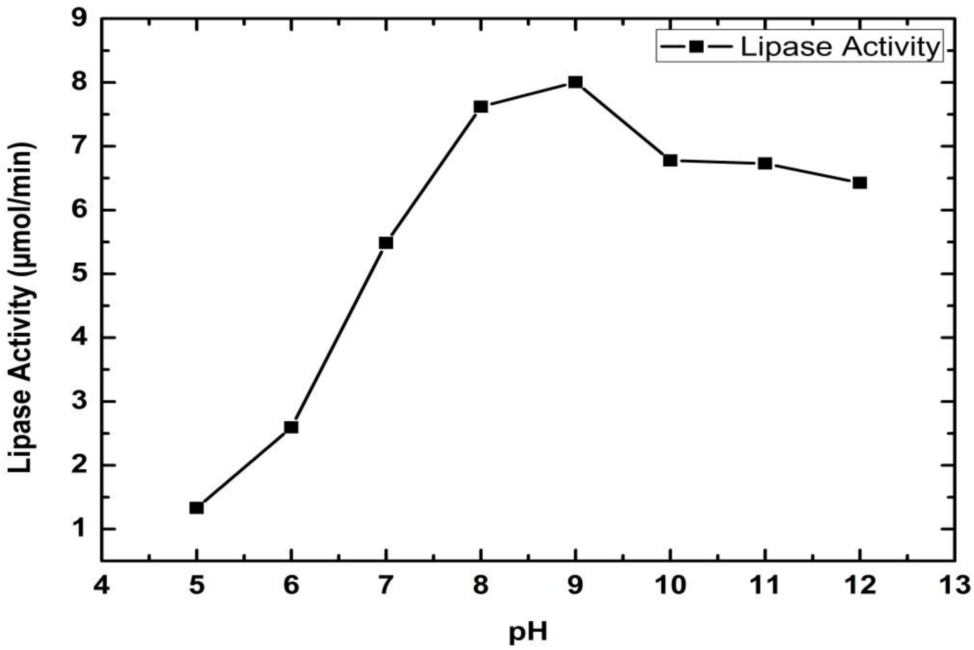


Figure 4.39: *Effect of pH on lipase activity*

The effect of pH was be measured by fixing the process variables substrate concentration at 420µM, enzyme concentration-200µl, incubation period for 10 mins and the optimum temperature at 45°C from the previous step. The lipase activity was measured at each pH 5-12. The maximum lipase activity of 8.005U was observed at pH 9.0 (Figure 4.39). Enzyme activity showed decreasing trend when increased above pH 9. It indicates that enzyme can act as an alkaline lipase. As the metabolic activities of the microorganisms were very sensitive to changes in pH and is found to be affected if pH level is higher or lower compared to the optimum value [Tembhurkar et. al. 2010].

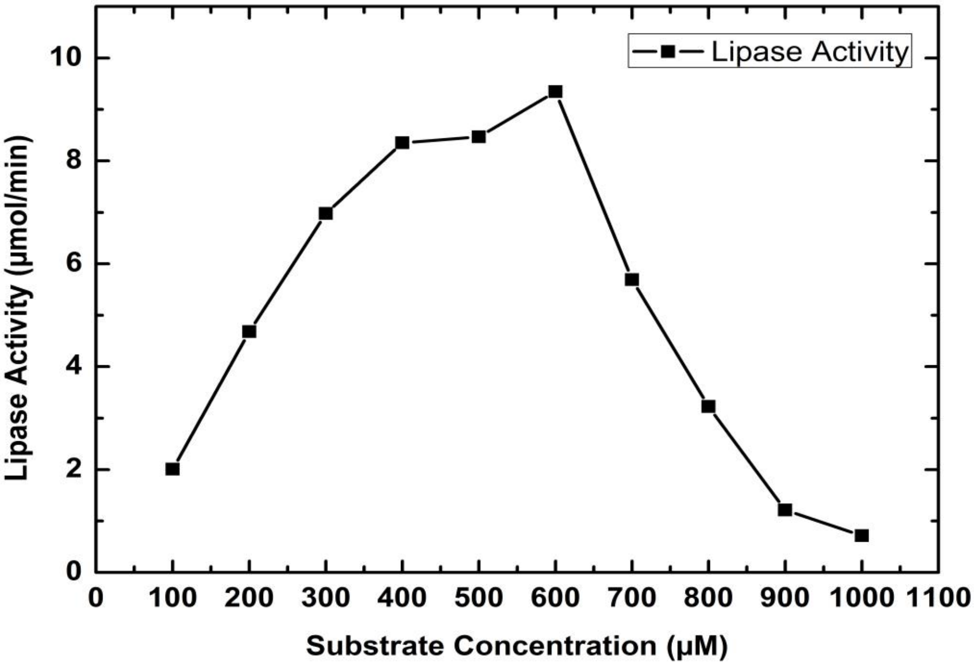


Figure 4.40: *Effect of Substrate concentration on lipase activity*

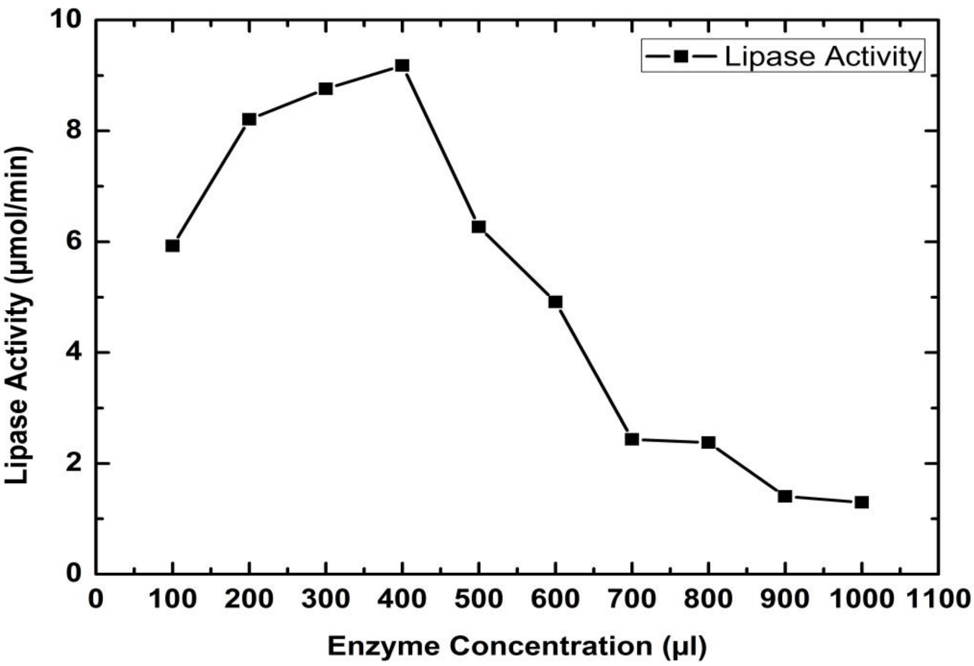


Figure 4.41: *Effect of Enzyme concentration on lipase activity*

The effect of Substrate concentration was measured by fixing the process variables enzyme concentration-200µl, incubation period for 10 mins and the optimum temperature and pH at 45°C and 9 from the previous steps. The lipase activity was measured at each Substrate concentration from 100-1000µM. The maximum lipase activity of 9.34Unit was observed at substrate concentration- 600µM (Figure 4.40). The lipase activity was gradually increasing up to optimum point there after suddenly decreasing its activity. It is attributed to higher the substrate concentration where more quickly product is produced (rate of reaction increases) until enzyme saturation is reached till substrate concentration has limited effect [Tembhurkar et. al. 2010].

The effect of enzyme concentration(in terms of volume) was measured by fixing the process variable incubation period for 10 mins and the optimum temperature at 45°C, pH at 9 and substrate concentration at 600µM from the previous steps. The lipase activity was measured at each enzyme concentration (in terms of volume) from 100-1000µl. The maximum lipase activity of 9.18 Unit was observed at enzyme concentration- 400µl (Figure 4.41). Increasing the enzyme concentration showed increase in enzyme activity. At high enzyme concentration thus is maximum contact between the enzyme and substrate molecules and form enzyme substrate complex and thus gives more activity at a certain time. However if we keep increasing the concentration, a point would be reached when the rate becomes constant and substrate molecules become the limiting factor [Sirisha et al. 2010].

The effect of Incubation time was measured by optimum process variable such as temperature-45°C, pH- 9, substrate concentration 600µM, enzyme concentration 400µl from the previous steps. The lipase activity was measured at each time of incubation from 5-30 min. The maximum lipase activity of 10.18 Unit was observed at incubation time-10 min (Figure 4.42). The longer an enzyme is incubated with its substrate, the greater the amount of product that was formed. However, the rate of formation of product is not a simple linear function of the time of incubation. So activity of enzyme should decreasing trend after certain time [Sirisha et al. 2010].

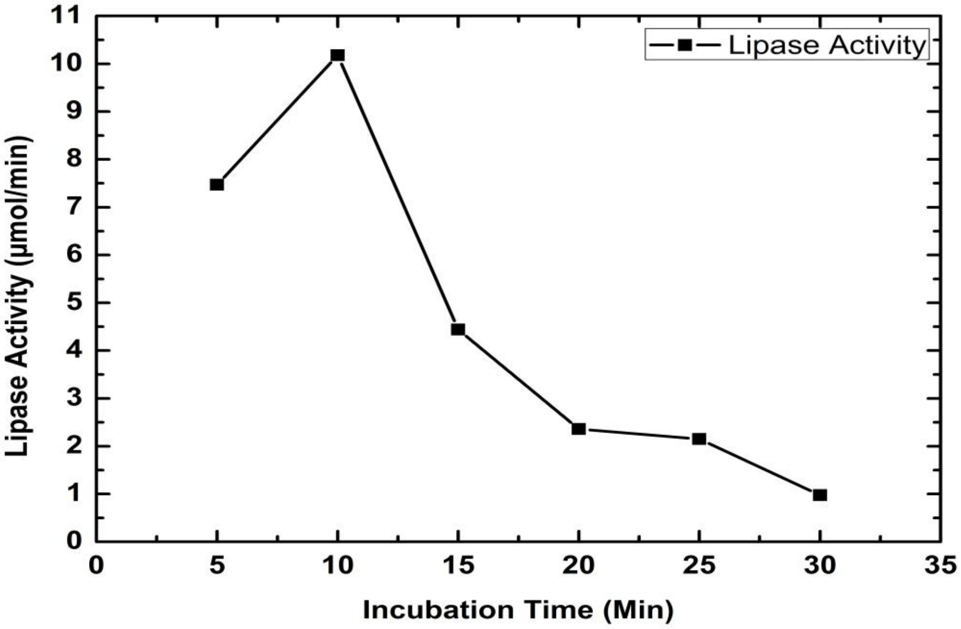


Figure 4.42: *Effect of Time of incubation on lipase activity*

4.9 Isolation of Lipase gene approach:

4.9.1 Estimation of DNA:

Genomic DNA was isolated from *Pseudomonas aeruginosa* is aerobic process condition by CTAB/Nacl method and purified by phenol-chloroform treatment method. The quantity and purity was checked by spectrophotometer and agarose gel electrophoresis.

4.9.1.1 Estimation of DNA by spectrophotometric method:

The Genomic DNA isolated from *Pseudomonas sp*. was having A_{260}/A_{280} ratio and was estimated 1.625. The ratio shows that the DNA was approximately free from protein contamination. The concentration of DNA was also estimated as per the mentioned formula.

DNA concentration (µg /ml) = OD at 260 nm × dilution times × standard value

= 0.013 × 20 ×50

= 13 µg /ml

4.9.1.2 Estimation of DNA by agarose gel electrophoresis:

1% agarose gel stained with ethidium bromide (0.5µg/ml) was used for quantification of DNA. The dye Ethidium bromide dye was used to make DNA bands visible in gel electrophoresis. It's a highly carcinogenic dye that penetrates Hydrogen bonds of DNA and fluoresces under UV light.

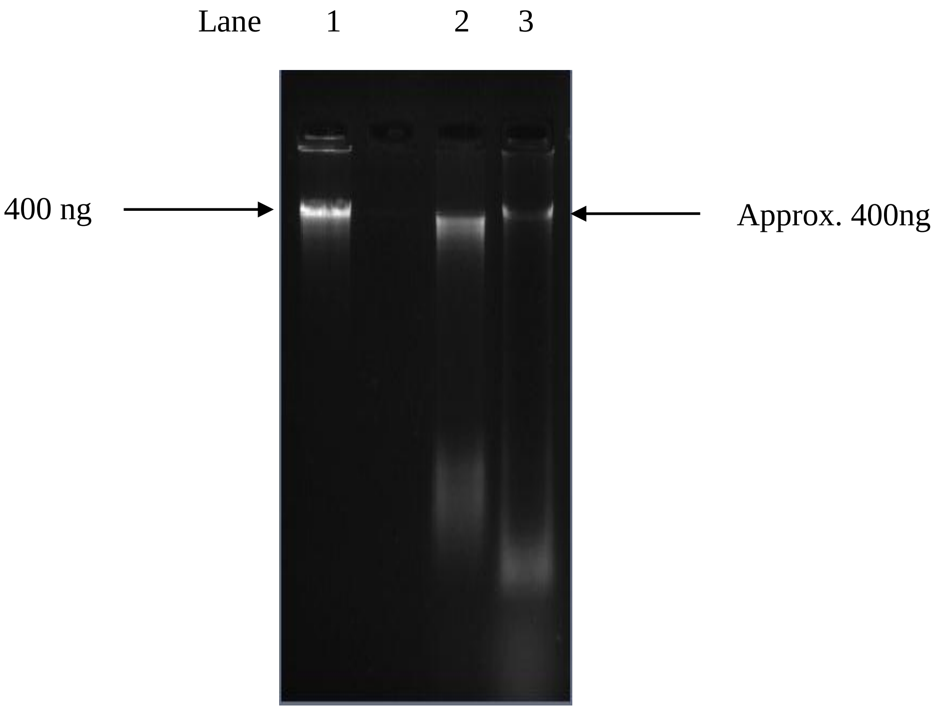


Figure 4.43: *Visualization of DNA under gel electrophoresis*

Lane-1: Marker DNA (400ng)

Lane-2 &3: Isolated DNA sample

In agarose gel the well was loaded with DNA along with gel loading dye. In lane-1 the marker DNA standardd reference (400ng) was loaded where as in Lane-2 & 3, DNA sample was loaded. From the figure 4.42 it can be seen that the sample DNA loaded has concentration of 400ng approximately.

4.9.2 PCR amplification of lipase gene:

The gene responsible for the production of lipase enzyme by PCR method was studied. The primer used in this method was Forward Primer: 5'CATATGATGAAAKGCTGYCGGGT-3' and Reverse primer: 5'GGATCCTTAAGGCCGCAARCTCGCCA-3' [Sifour et. al. 2010].

The annealing temperature profile for PCR was calculated using primer melting temperature [Appendix-J].

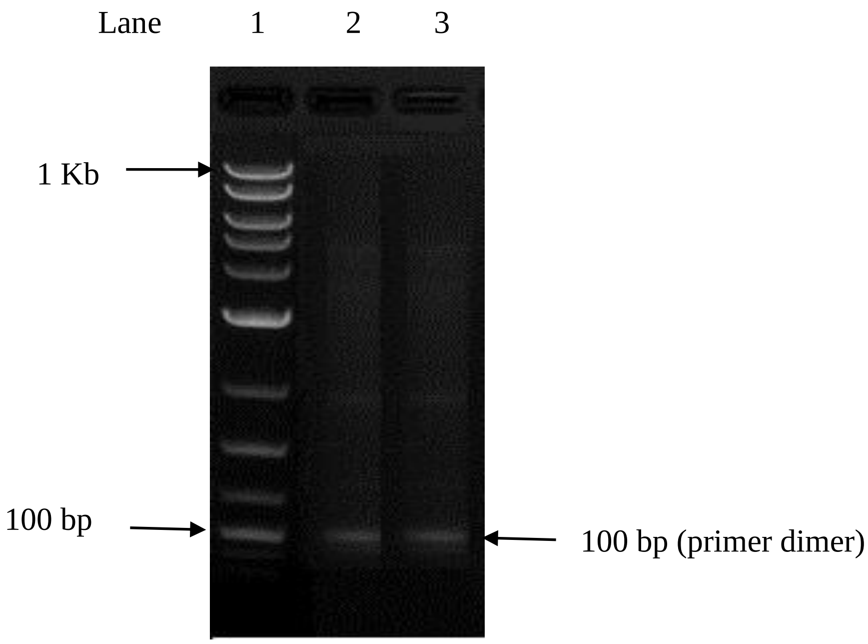


Figure 4.44: *Gel image of PCR product*

Lane-1: Marker DNA (1 kb), Lane-2 &3: PCR product

It can be observed that the gene was not amplified properly by PCR which can be attributed to primer dimer problem. Primer dimer(PD) of primer molecules revealed attachment (hybridized) to each other because of strings of complementary bases in the primers. PD shows very light coloured band in low base pair region. The failure of PCR amplification based on the report mentioned for *Bacillus stearothermophilus* did not respond well to our lipase gene isolated from *Pseudomonas aeruginosa*which [Sifour et. al. 2010]. Primer selection for *Pseudomonas aeruginosa* was based on the enzyme family history. It was already reported that *Bacillus stearothermophilus* also belong to lipolytic enzyme family. However our primer selected did not respond to the PCR amplification of this gene which can be attributed to the difference in sub family of the lipolytic enzyme family.

4.9.3 Isolation of lipase gene by Recombinant DNA technology:

Due to failure of PCR amplification method, recombinant DNA technology was used for the isolation of lipase gene by cloning method. This method was followed by restriction digestion, ligation and transformation. The reported result was shown in various figures (4.45-4.48)

4.9.3.1 Restriction digestion:

Restriction digestion was carried out by using restriction enzyme (BAM Hl, Thermo scientific). The restriction digestion of DNA sample was carried out for four different hours (0hr, 3hr, and 6hr, overnight) at 37^0C and loaded on to agarose gel electrophoresis and was shown in figure 4.45.

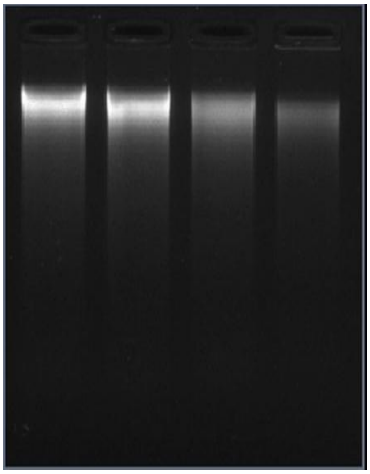


Figure 4.45: *Gel image of Restriction digestion*

Lane-1: 6hr sample, Lane-2: Overnight sample, Lane-3: 3hr sample, Lane-4:0hr sample

It can be observed that overnight incubated sample showing maximum digestion by comparing band intensity of different hour sample. Overnight incubated digestion sample was chosen for further procedure (ligation & transformation).

4.9.3.2 Ligation and Transformation:

Ligation was carried out using *T4 DNA Ligase* (Thermo scientific) enzyme in pUC-19 vector. The reaction was carried out at 22^0C for one hour. Further the ligated sample was proceed for Transformation.

Transformation was carried out using bacterial aid transformation kit (Thermo scientific). It was carried out by following manufacture's transformation protocol. *E.coli*

(DH5-α) cells were used as a competent cell for this study. 100 pg of supercoiled (pUC-19) DNA was taken as control for transformation study. The result was shown in Figure 4.47.

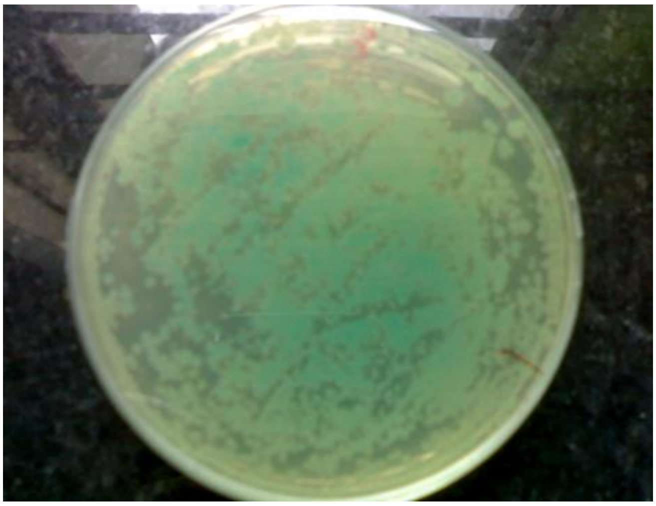


Figure 4.46: *growth of Control in transformation*

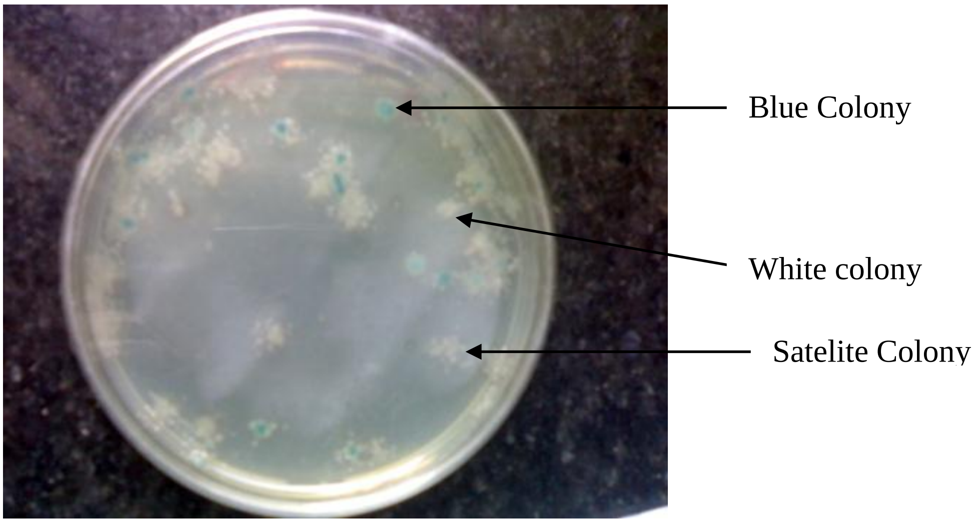


Figure 4.47: *Blue and white colony selection in transformation*

The control was taken for transformation study indicates the growth of blue colony, the plasmid (Figure 4.46). The white colony represents the the growth of transformant whereas the small colony indicates the growth of non-transformants (satellite colony)[figure 4.47]. The transformation efficiency of our product is 5×10^5.

All the Cloned products (Cells) were checked for lipase activity by substrate agar plate method forming zone of diffusion (Figure 4.48).

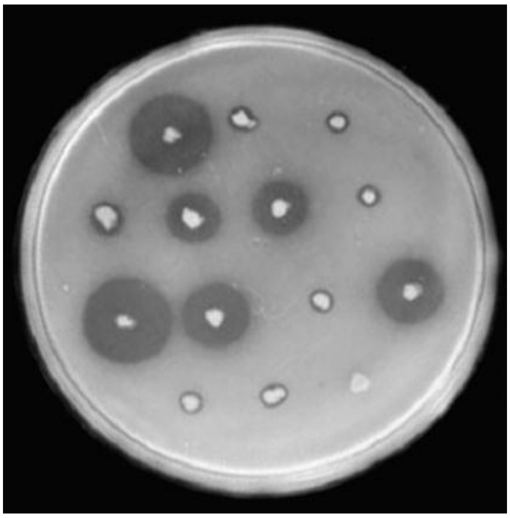


Figure 4.48: *Cloned product lipase activity*

Thus our observation indicates the occurrence of gene responsible for lipase activity. The gene responsible for lipase activity was isolated and undergone for colony PCR. Colony PCR did not respond for gene amplification due to gene size may more than 4kb. However the identification of gene is recommended for future scope of research. All the cloned product observed in figure 4.47 is stored by constructing genomic DNA library and may use for further research.

The E.coli cloned product which confirms the lipase activity can be used for biotransformation study instead of *Pseudomonas aeruginosa.* It is having doubling time of 4 hours in anaerobic condition whereas the doubling time of *E.coli* is 20 mins. The E.coli cloned product can be used instead of *Pseudomonas aeruginosa* for further biotransformation process which may reduce the time period for biotransformation process.

4.10 Antimicrobial activity of Lauric acid:

The antimicrobial activity of Lauric acid was tested with two bacterial strain *E.Coli* (Gram -ve) and *Bacillus Subtilis* (Gram +ve). Various amount of lauric acid (0.2, 0.4, 0.6, 0.8& 1ml) were dissolved in 5%DMSO. 1ml of bacterial suspension was considered as positive control and 1 ml of 5% DMSO [Georgiana et. al. 2012] was considered as negative control. The antimicrobial activity of lauric acid was shown in figure 4.49 and 4.50 by bacterial growth curve.

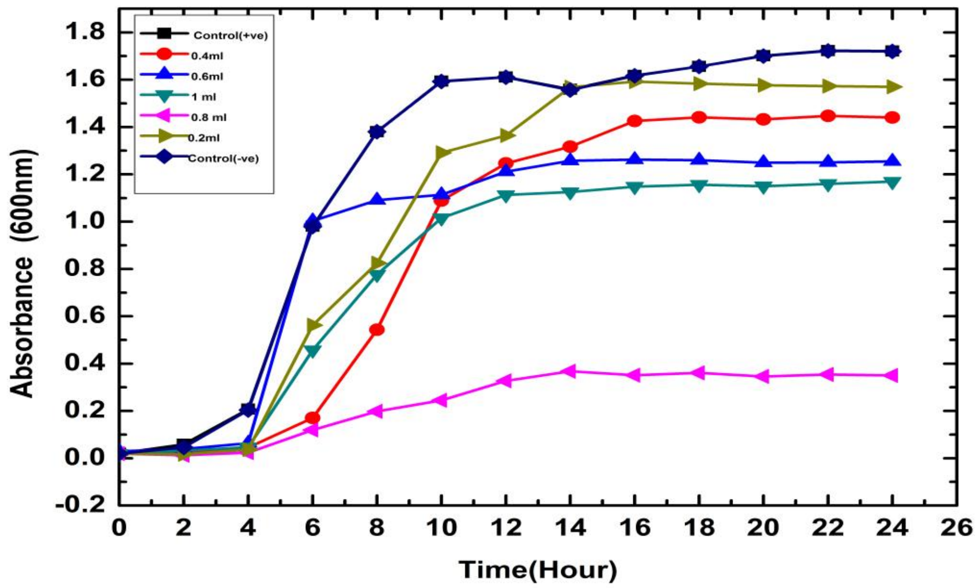


Figure 4.49: *Antimicrobial activity of Lauric acid against E.coli*

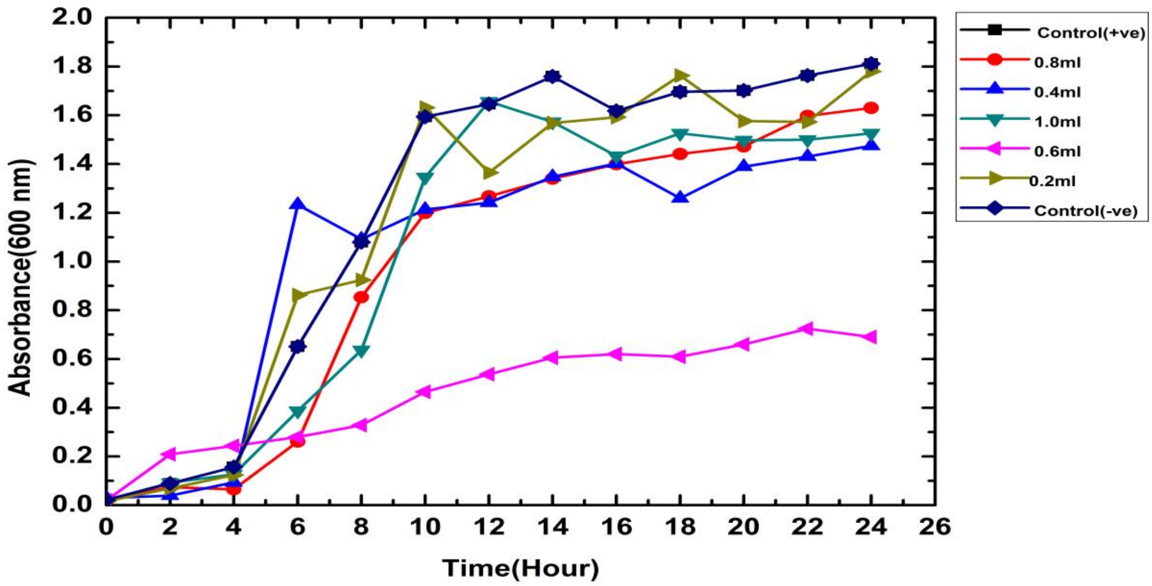


Figure 4.50: *Antimicrobial activity of Lauric acid against B. subtilis*

Maximum growth inhibition of lauric acid occurs at 0.8ml in *E.coli* (Figure 4.48) and 0.6ml in *B. subtilis*(Figure 4.49).

CHAPTER-5

CONCLUSION & FUTURE WORK

5. Conclusion:

- Eight microbial stains were isolated from various sites namely local oil shop, garden Soil and Gokharkuda beach (Rushikulya river) that showed existence of these species on oil source.
- Among the eight isolates one of the bacterial strain have shown very good results on coconut oil biotransformation.
- The morphological, physiological and biochemical tests shows that bacterial stain ‘GB-1’ is *Pseudomonas sp.*
- 16s-rDNA analysis & phylogenetic analysis confirmed the organism *Pseudomonas aeruginosa.*
- Biotransformation study of coconut oil by the strain *Pseudomonas aeurignosa was* conducted in both aerobic and anaerobic environment and anaerobic condition proved to be most suitable condition than other.
- In the single step bio-transformed process, coconut oil was transformed in to Fatty acids and detected by GC-MS analysis quantitatively cum qualitatively.
- Optimization of process parameters like Temperature, pH, substrate concentration and agitation (RPM) source were conducted by Taguchi method. Optimum process parameters were Temperature-40^{0}C, optimum pH-8, optimum substrate concentration-2% and RPM-150.
- Lab scale bio fermenter study for lauric acid production was conducted for eight days using optimum process parameter and GC MS analysis it indicates that that 90% of Glycerol trilaurate content was utilized by the bacteria for biotransformation to Lauric acid (89.68%) and traces of myristic acid and solvent. It was compared with literature and showing good agreement with those reported(Krishna et. al. 2010).
- Experimental result of product kinetics were fitted and agreed with to the second order model equation.
- Continous stirred tank reactor was preferred than plug flow reactor based on space time. The effective reactor liquid volume for CSTR is 4375 litre.

- Lipase activity was detected by substrate agar plate and rhodamine-B dye agar plate method. Estimation of lipase activity was carried out by standard p-Nitrophenol method.
- Lipase activity estimation was carried out on daily basis for both aerobic and anaerobic process condition upto 8 days. Aerobic condition showing maximum lipase activity than compared to anaerobic condition of 8.75 Unit in 96 hours whereas anaerobic condition showing highest activity of 0.97 Unit in 120 hours.
- Optimization for maximum lipase activity was conducted by taking process parameters such as Temperature, pH, Enzyme concentration, substrate concentration and time of incubation were optimized as 45^0C, 9, 400µl, 600µM and 10 min respectively based on their maximum lipase activity.
- Lipase gene was isolated by cloning technology (Restriction digestion, Ligation and transformation method with vector PUC-19 digested with Bam H1 and inserted in E.coli Dh5α Culture and confirmed by the substrate agar plate method based on zone diffusion.
- Genomic DNA library was constructed by using lipase gene along with other gene and stored for further studies.

Future work:

- To isolate and select the other microbes or enzymes which can transform the oil into products like fatty acids.
- To screen other natural oils for fatty acid production using the microbe/enzymes isolated.
- To study the pathway for Biotransformation in both aerobic and anaerobic (pH, Temperature, Nutrients).
- Developing the downstream processing steps for biotransformed product.
- To isolate the crude enzyme and protein estimation.

CHAPTER-6

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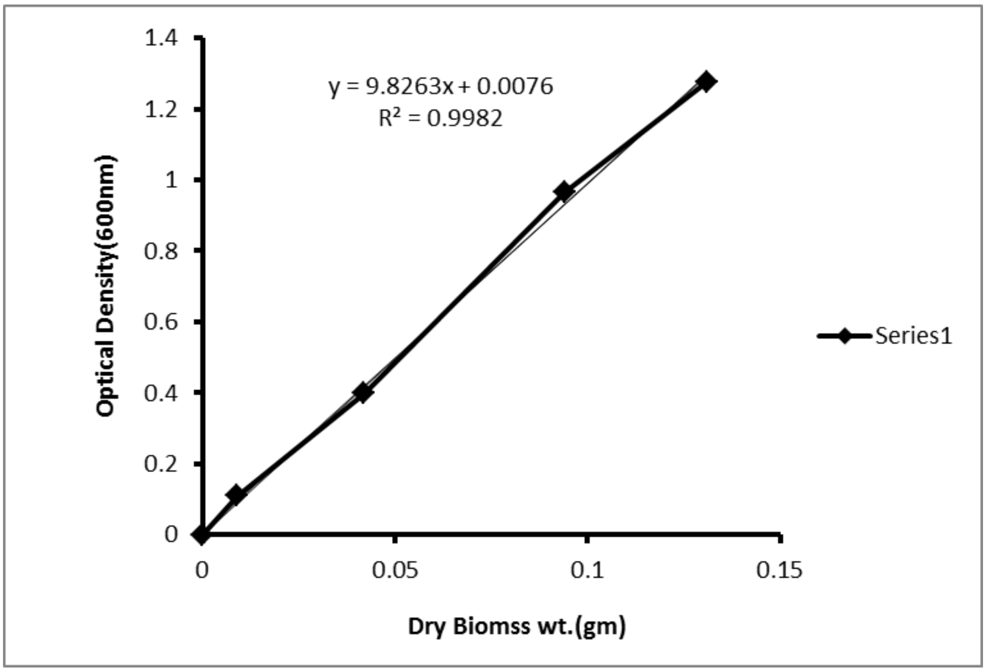
APPENDIX

APPENDIX

Appendix-A

Biomass Standard graph



The different biomass weight was measured after drying the sample by taking the difference between final and initial weight. From the above equation the biomass weight of other unknown sample was calculated.

Appendix-B

Composition of Luria Bertani broth

Ingredients	Gms / Litre
Casein enzymic hydrolysate	10.000
Yeast extract	5.000
Sodium chloride	5.000
Final pH (at 25°C)	7.0±0.2

Suspend 20 grams in 1000 ml distilled water. Heat to dissolve the medium completely.

Appendix-C

Lipase activity assay composition

	Substrate (μl)	Enzyme (μl)	Buffer (μl)	Incubation Time (10 Min)	Triton-x100 (μl)	1M Na_2CO_3 (ml)
S^+E^-	100	0	1850		50	1
S^-E^+	0	200	1750		50	1
S^+E^+	100	200	1650		50	1

Reagent blank (A & B) = 1950 μl buffer + Triton-X 100(50μl) + 1M Na_2CO_3 (1 ml)

S^+E^- : Substrate blank or only substrate

S^-E^+ : Enzyme Blank or only enzyme

S^+E^+ : Test(Both substrate and enzyme)

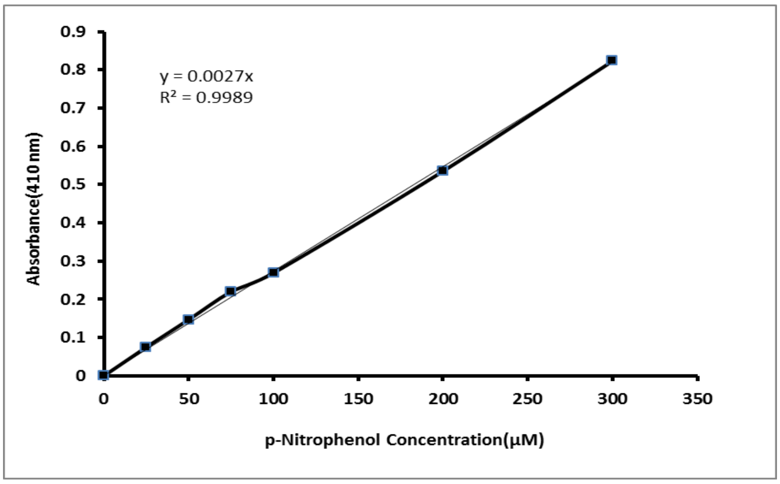
For each sample 3 different readings were taken as per above table. The final absorbance reading for each sample was calculated as following:

Final absorbance reading= $[(S^+E^+)-(S^+E^- + S^-E^+)]$

The spectrophotometer reading was taken at absorbance 410 nm.

Appendix-D

p-nitrophenol standard graph



One unit of enzyme activity (IU) was defined as the amount of enzyme that liberated 1 μmol of p-nitrophenol per minute under the standard assay conditions.

Appendix-E

Biuret method of protein estimation

This is one of the standard and common method for estimation of protein.

Apparatus required: Test tubes, Beakers, Conical flask, Spectrophotometer

Reagents: protein solution (BSA), Biuret reagent

Preparation of Biuret reagent:

- 3 gms of $CuSO_4.5H_2O$ and 9 gms of Na-K Tartarate are dissolved in 500ml of 0.2N NaOH sol. 5 gms of KI was added and the volume was made to 1 lit .by adding NaOH solution.
- For 200 ml, accordingly 0.6gm of $CuSO_4.5H_2O$, 1.8 gm of Na-K tartarate,100ml of 0.2N NaOH solution.,1gm of KI
- For preparing 0.2N NaOH , 1.6 gm of NaOH was dissolved in 200 ml of distilled water
- Protein standard (0.5%)- 1 gm of BSA in 200 ml of distilled water

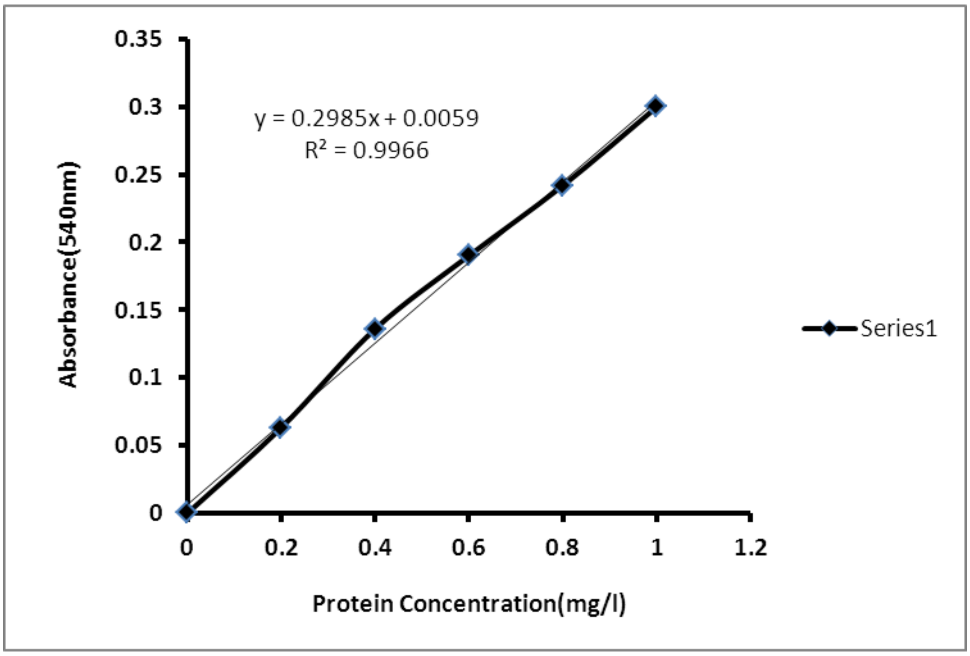
Principle: Biuret test is used for detecting the presence of peptide bonds. It relies on the reduction of copper (II) ions to copper (I), the latter form a complex with the nitrogens of the peptide bonds in an alkaline solution. A violet color indicates the presence of proteins

Procedure:

- 1.5 ml of protein sol. was suspended in a test tube containing 1.5 ml of 1N NaOH heated for 4-5 min.
- 3 ml of biuret reagent was added to this mixture.
- The contents were mixed and incubated in a water bath set at 37^{o}C for 10 min.
- The test tube was cooled at room temp.
- Optical density was measured at 540nm in a spectrophotometer
- A standard curve was prepared by taking different conc. of BSA protein solutions vs Absorbance (OD).

Appendix-F

Protein Standard graph



The standard graph of protein was prepared by taking different conc. of BSA protein solutions Vs. Absorbance (OD). The amount of lipase (Concentration) produced in anaerobic condition was measured by using this graph.

Appendix-G

Preparation of TE Buffer:

- 100mM of Tris HCL (pH-8.0)+10mM EDTA(pH-8.0)
- To 1.21g of Tris Cl was dissolved with 0.372g of EDTA and the volume was made up to 100ml after adjusting the pH 8.0.

Preparation of CTAB-NaCl Solution:

To 4.1g of NaCl, 80ml of water and 10g of CTAB was added to it while heating and stirring continuously. It was heated up to 65oC to dissolve and later the volume was adjusted to 100ml.

Preparation of Tris Saturated Phenol:

- Phenol was melted at 68oC and hydroquinoline was added to a final concentration of

0.1%.

- Equal volume of 1M Tris-Cl was added and the mixture was added for 15 minutes.
- The upper aqueous layer was removed.
- The above two steps were repeated with the lower layers with 1M Tris (pH-8.6) and finally with 0.5M Tris (pH-8.6) for 2-3 times until the pH of the phenol reaches 8.0.
- 0.1M Tris (equal volume) of pH 8.0 was added to phenol containing 0.2% β-mercaptoethanol and stored in dark amber colored bottle at 4^oC.

Appendix-H

Preparation of Different pH buffers

Preparation of Citric acid/ sodium citrate buffer (0.1M, pH-5):

It was prepared by taking 39ml of 0.1M citric acid in 100ml volumetric flask(100ml) and was mixed with 0.1M trisodium citrate.

Preparation of Citrate buffer (0.1M, pH-6):

It was prepared by taking 0.1M tris sodium citrate of 86ml and 0.1M citric acid of 14 ml in a volumetric flask (100ml) and was mixed properly.

Preparation of Phosphate buffer (0.1M, pH-7):

It was prepared by taking 0.1M disodium hydrogen phosphate of 57.7 ml and 0.1M of monosodium phosphate of 42.3 ml in a volumetric flask(100ml) and was mixed properly.

Preparation of Tris-Hcl buffer (0.1M, pH-8.0):

It was prepared by taking 0.1M Tris of 71 ml and 0.1M of hydrochloric acid (Hcl) of 29ml in a volumetric flask (100ml) and was mixed properly.

Preparation of Carbonate buffer (0.2M, pH-9.2):

It was prepared by taking 0.2M sodium carbonate of 10ml and 0.2M sodium bicarbonate of 90 ml in a volumetric flask (100ml) and was mixed properly.

Preparation of Carbonate buffer (0.2M, pH-10.0):

It was prepared by taking 0.2M sodium carbonate of 55 ml and 0.2M sodium bicarbonate of 45 ml in a volumetric flask (100ml) and was mixed properly.

Preparation of buffer (pH-11.0):

It was prepared by taking 0.05M of disodium hydrogen phosphate of 91.8 ml and 0.1M sodium hydroxide of 8.2 ml in a volumetric flask (100ml) and was mixed properly.

Preparation of buffer (pH-12.0):

It was prepared by taking 0.05M of disodium hydrogen phosphate of 46.2 ml and 0.1M sodium hydroxide of 53.8 ml in a volumetric flask (100ml) and was mixed properly.

NOTE: All the buffers were prepared by adjusting pH with 1N hydrochloric acid (Hcl) and 1N Sodium hydroxide (NaoH).

Appendix-I

Composition of PCR solution

Chemical composition	Quantity(μl)
ddH_2O	19.13
Taq buffer	2.5
Primer F	1
Primer R	1
Taq pol.	0.17
dNTP	0.2
DNA	1
Total	**25**

Appendix-J

Annnealing Temperature of PCR Cycle [web ref. 12]

$T_A = T_M - 5^0C$

T_M = Melting Temperature

T_A = Annealing Temperature

The melting temperature can be calculated as from following formula:

Melting Temperature (T_M) = 2(A+T) + 4(G+C) [A+T content and G+C content can be calculated from primer]

For our study the melting temperature of our primer is

Forward primer melting temperature (T_{MF}) = 57.5^0C

Reverse primer melting temperature (T_{MR}) = 64.5^0C

So the average of melting temperature is $T_M = (T_{MF} + T_{MR} /2) = (57.5+64.5/2) = 61^0C$

The formula for Annealing Temperature is $T_A = T_M - 5^0C$

Now Annealing Temperature (T_A) = $61^0C - 5^0C = 56^0C$.

So, the annealing temperature for our study is 56^0C.

Appendix-K

Different recipes for transformation

Ampicillin stock solution (50mg/ml):

2.5gm of ampicillin sodium salt was dissolved in 50 ml of deionized water. It was filter sterilized and stored as aliquots at -20^0C.

X-gal stock solution (20mg/ml):

200mg of X-gal(5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) was dissolved in 10 ml of dimethylsulfoxide(DMSO) and stored at -20^0C in dark place. 40µl of solution was spread before the transformation on to the surface of LB antibiotic agar plates.

IPTG solution (100mM):

1.2 gm of IPTG (Isopropyl-b-D-thiogalactopyranoside) was dissolved in 50ml of deionized water. It was filtered sterilized and stored at -20^0C. 40µl of solution was spread before the transformation on to the surface of LB antibiotic agar plates.

LB (Luria-Bertani) antibiotic plates:

LB plates were prepared by using LB agar. 1ml of ampicillin stock solution(final concentration 50µg/ml) was spread on to the plates.

Transformation efficiency:

Transformation efficiency was calculated using following formula:

[(Cfu on control plate/ 0.1ng of control DNA plated) * 1000ng/µg].

Appendix-L

Compound percentage calculation in GC

The Percentage of each compound was calculated using this formula from peak area.

Each compound (%) = [(Peak area of that particular compound/ Total peak area) * 100]

Appendix-M

Biotransformation study efficiency calculation

The biotransformation efficiency was calculated based on initial and final carbon source utilization by using following formula:

$$\text{Biotransformation Effeciency} = \frac{\text{Final Product (gms)}}{\text{Total carbon source used (gms)}} \times 100$$

$$= \frac{0.746}{1.85} \times 100$$

$$= 40.32\%$$

Appendix-N

Detailed procedure of *16s rDNA* molecular characterization

The following detailed procedure of 16S rDNA analysis was incorporated in materials and methods section:-

For DNA Isolation Bacterial DNA isolation Kit was used (Xcelgen):

Quantitation and Quality Assessment of DNA:

The DNA stock samples was quantified using Nanodrop spectrophotometer at 260 and 280 nm using the convention that one absorbance unit at 260 nm wavelength equals 50 µg DNA per ml. The Ultra violet (UV) absorbance was checked at 260 and 280 nm for determination of DNA concentration and purity. Purity of DNA was judged on the basis of optical density ratio at 260:280 nm. The DNA having ratio between 1.8 to 2.0 was considered to be of good purity. Concentration of DNA was estimated using the formula.

Concentration of DNA (mg/ml) = OD 260 x 50 x Dilution factor

Quality and purity of DNA were checked by agarose gel electrophoresis. Agarose 0.8% (w/v) in 0.5X TAE (pH 8.0) buffer was used for submarine gel electrophoresis. Ethidium bromide (1%) was added @ 10µl /100ml. The wells were charged with 5µl of DNA preparations mixed with 1µl gel loading dye. Electrophoresis was carried out at 80V for 30 min at room temperature. DNA was visualized under UV using UV transilluminator. The DNA was used further for PCR.

Polymerase Chain Reaction:

16S RNA gene fragment was amplified by PCR from genomic DNA using 16S gene universal primers: 8F and 1492R

Details of 16S Universal Primer Sequence

8F: 5'AGAGTTTGATCCTGGCTCAG3'

1492R: 5' ACG GCT ACC TTG TTA CGA CTT 3'

PCR was carried out in a final reaction volume of 25 µl in 200 µl capacity thin wall PCR tube in Eppendorf Thermal Cycler. Composition of reaction mixture for PCR is given in Table 1. PCR tubes containing the mixture were tapped gently and spin briefly at 10,000 rpm. The PCR tubes with all the components were transferred to thermal cycler. The PCR protocol designed for 30 cycles for the primers used is given in second Table.

Composition of reaction mixture for PCR

Components	**Quantity**	**Final**
DNase-RNase free water	7.50 µl	--
2X PCR Master mix(MBI Fermentas)	12.50 µl	1X
Forward primer(10 pmole/µl)	1.00 µl	10 pmole
Reverse primer(10 pmole/µl)	1.00 µl	10 pmole
Diluted DNA(30ng/ µl)	3.0 µl	----
Grand Total	**25 µl**	

Steps and conditions of thermal cycling for PCR

Steps	**Temperature**	**Time**	**Cycles**
Initial Denaturation	°C	in	1
Final Denaturation	°C	Sec	30
Annealing	°C	Sec	
Extention	°C	Sec	
Final Extention	°C	min	1

Visualization of PCR Product:

To confirm the targeted PCR amplification, 5 µl of PCR product from each tube was mixed with 1 µl of 6X gel loading dye and electrophoresed on 1.2 % agarose gel containing ethidium bromide (1 per cent solution @10 µl/100 ml) at constant 5V/cm for 30 min in 0.5 X TAE buffer (Gel Image). The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system (Biorad).

Purification of PCR product:

Amplified PCR product was purified using Qiagen Mini elute Gel extraction kit according to the manufactures protocol.

Sequencing of Purified DNA:

Sequencing of Purified 16S rDNA Gene Segment

The concentration of the purified DNA was determined and was subjected to automated DNA sequencing on ABI 3730xl Genetic Analyzer (Applied Biosystems, USA). Sequencing was carried out using BigDye® Terminator v3.1 Cycle sequencing kit following manufacturers instructions.

Cycle Sequencing:

Cycle sequencing was performed following the instructions supplied along with BigDye® Terminator v3.1 Cycle Sequencing Kit. The reaction was carried out in a final reaction volume of 20µl using 200µl capacity thin wall PCR tube. The cycling protocol (Table 4) was designed for 25 cycles with the thermal ramp rate of 1°C per second.

Cycling protocol for sequencing reaction

Step	Temperature	Time
Denaturation	96°C	10 sec
Annealing	52°C	5 sec
Extension	60°C	4 min

* Repeat step 1 to 3 for 25 cycles

After cycling, the extension products were purified and mixed well in 10 µl of Hi-Di formamide. The contents were mixed on shaker for 30 minutes at 300xg. Eluted PCR products were placed in a sample plate and covered with the septa. Sample plate was heated at 95°C for 5 min, snap chilled and loaded into autosampler of the instrument.

Electrophoresis and Data Analysis:

Electrophoresis and data analysis was carried out on the ABI 3730xl Genetic Analyzer using appropriate Module, Basecaller, Dyeset/Primer and Matrix files.

Sequence Analysis:

- Both ends of the sequence was verified with the chromatogram file and edited if required. The sequence was converted into fasta format.
- The 16S rDNA gene sequence were used to carry out BLAST (Basic local Alignment Search tool) with nr database of NCBI Genbank using MEGABLAST algorithm.
- The BLAST data was arranged in maximum percentage identity and first ten sequence was selected and exported in FASTA format.
- Based on maximum identity score and query coverage the best highly identical 10 sequences were selected and aligned using multiple alignment software program ClustalW(MEGA4 tool).
- The evolutionary history was inferred using the Neighbor-joining method. The bootstrap consensus tree inferred from 500 replicates is taken to represent evolutionary history of the taxa analyzed.
- The evolutionary distances were computed using the Kimura 2- parameters method. Phylogenetic analysis was conducted in MEGA4.

Publication

Conference Paper

 V.B.Patro, S.Mishra, Comparison of Biotransformation process in natural oil in International Conference of Association of microbiologists of India-2012,Bhubaneswar, India.

 V.B.Patro,S.Mishra, Lipase mediated Biotransformation process in natural oil in International Conference on Biotechnology and advances: omics way to approach-2012 ,Bhubaneswar, India.

 V.B Patro, S. Mishra, Microbial leaching for the extraction of Nickel and cobalt from chromite overburden of sukinda mines, odish in CHEMCON-2011, Bangalore, India.

Gene sequence submission(NCBI)

 V.B.Patro, S.Mishra, *Pseudomonas aeruginosa* strain named as VBP-01, Genebank accession number-KC310862.

Curriculum vitae

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OBJECTIVE

To use my knowledge in best possible way to effectively deliver results in a challenging position as Professional Engineer in a reputed firm.

EDUCATIONAL QUALIFICATIONS

Master of Technology; (National Institute of Technology, Rourkela 2011 -2013)

- Specialization: Chemical Engineering(Biochemical Engg & Environment Biotechnology)
- C.G.P.A: 8.86(Upto 3rd Sem)

Bachelor of Technology; (Bharath University 2006 - 2010):

- Specialization: Industrial Biotechnology
- C.G.P.A: 8.32

12th Standard (C.H.S.E– 2006):

- Maharishi College of Natural Law, Bhubaneswar.
- Marks Percentage: 52.6%

10th Standard (B.S.E - 2004):

- Swami Chidananda high School, Turumu, Odisha.
- Marks Percentage: 74.0%

OTHER ACADEMIC HIGHLIGHTS

Project / Seminar:

- *Currently doing work on "Biotransformation of natural oil into industrial useful product".*
- Done project on "*Microbial leaching for the extraction of Nickel and Cobalt from the Chromite overburden of sukinda mines Odisha"*at IMMT(CSIR), Bhubaneswar, Odisha.
- Done project on *"Production of Biofuel from germinated maize seeds"* at Bharath University,Chennai.

Publications /Conférences :

- Paper accepted and presented in **CHEMCON-2011,** organized by Indian Institute of Chemical engineers(IIChE),Bangalore region on "*Microbial leaching for the extraction of Nickel and Cobalt"* at Annual IIChE Meeting 2011, Bangalore, India.
- Presented a paper on "*Controlling the corrosion of Carbon steel in rain water using environmental friendly inhibitors*" in National Seminar On Environmental Biotechnology, Department of Biotechnology, Kumara guru college of Technology, Coimbatore-***1st prize***.
- Presented a Poster on "*Application of Nanocrystalline Materials for Solar cell*" in National level Technical symposium, Department of Chemical Engineering, Coimbatore Institute of Technology, Coimbatore-***2nd prize***.
- Presented a paper on *"Effect of Nutrient concentration and chlorophyll content of Black gram treated with Oscillatoria sp. in organic manures under drought stress"* in International Conference on Biotechnological solutions for Environmental sustainability (Icon-BSES), School of Bio Sciences and Technology, VIT University, Vellore.

Scholastic Achievements :

- IELTS qualified with overall band score 6.0(2010)

OTHER CREDENTIALS & EXPERIENCE

- Attended a workshop regarding "*Bioprocess engineering and fermenter operation*" at Indian institute of technology Madras.
- Industrial training on *"Effluent Treatment"* in Technical Services at Indian Rare Earth ltd.(IREL), OSCOM, Matikhalo (Ganjam dist.), Odisha.
- Attended a workshop on *"Human DNA finger printing (ALU Typing)"* at Bharath University.
- Attended a Short-term training course on *"Vaccinology and water analysis"* at Pasteur Institute of India, Coonoor, The Nilgiris, Tamilnadu.
- Attended a Short term training course on *"Biofuel (Biodiesel & Bioethanol) production"* at Kalinga plant resource center, Bhubaneswar, Odisha.
- Done Implant Training on *"Alcohol Fermentation of sugarcane Molasses using Saccharomyces Cerevisiae"* at Aska Co-operative Sugar Industries Ltd., Aska, Odisha.

EQUIPMENTS HANDLED & SKILLS

- Major instruments like BET, TGA,GC, HPLC, TOC, IR Spectrometer, Fermenter, Sonicator, Viable cell analyzer, Biochemical analyzer etc.
- Chromatography, Microbiology, Enzymatic, upstream and downstream processing techniques
- Microsoft Office, Minitab, Spectrum, Image J, Origin.

EXTRA-CURRICULAR ACTIVITIES

- Team leader of Biofuel project from germinated maize seeds at Bharath University, Chennai.
- Student Coordinator of "Biovision" at Bharath University, Chennai held in 2009.
- Student Coordinator of "National Students Convention" at National Institute of Technology, Rourkela held in September 2011.
- Student Coordinator of "RACEE" at National Institute of Technology, Rourkela held in January 2012.
- Won 1st prizes in State and District level Science exhibition.
- Won 2nd prize in "Chemi balloon" a Technical event organized by National Institute of Technology, Rourkela.